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Strategies for improving islet transplantation outcome

Rackham, Chloe

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Strategies for improving islet transplantation outcome

A thesis submitted by

Chloe Rackham

For the degree of Doctor of Philosophy
from the University of London

Diabetes Research Group
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Abstract

Allogeneic islet transplantation offers the possibility to treat selected patients with brittle Type 1 Diabetes Mellitus (T1DM). Limited availability of islets is a major obstacle to the more widespread use of islet transplantation as a therapy for the majority of patients with T1DM. This is exacerbated by extensive islet cell death during the early post transplantation period, which increases the number of islets required to achieve insulin independence. Additionally, disturbances to normal islet architecture and morphology, as well as suboptimal vascular engraftment during the post transplantation period, contribute to the long term decline in graft function. As well as substantial stresses to the islets during the post transplantation period, functional β -cell mass is lost during pre-transplant culture, further contributing to the inefficient use of valuable donor islets. Mesenchymal Stem Cells (MSCs) secrete a number of soluble trophic factors to affect neighbouring cells, making them excellent candidates for improving the survival of islet cells during culture and after transplantation. The overall aims of the studies described in this thesis were to investigate strategies to improve islet transplantation outcome. Using a syngeneic minimal islet mass model, it was demonstrated that MSC co-transplantation improved the rate and number of streptozotocin-induced diabetic mice attaining normoglycaemia by one month. The beneficial influence of MSCs was attributed to the maintenance of normal islet size and morphology, as well as increased rate and overall extent of islet revascularisation. Additional studies demonstrated that dispersing islets beneath the kidney capsule of diabetic mice produced superior transplantation outcome to that of islets which were implanted as a single pellet, confirming the importance of maintaining normal islet size and morphology at the implantation site. Preculturing islets with MSCs was shown to enhance functional β -cell mass *in vitro*, which correlated with the better function of grafts consisting of MSC precultured islets compared to islets cultured alone, *in vivo*. In summary, MSC-based strategies offer great potential for enhancing the efficiency of clinical islet transplantation, which may help in achieving its more widespread application to the majority of patients with T1DM.

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List of abbreviations

Ab	Antibody
α -SMA	alpha-smooth muscle actin
ATP	Adenosine triphosphate
BM	Bone marrow
bp	Base Pair
BS-1	Bandeiraea simplicifolia
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium ions
C57BL/6	inbred mouse strain C57 black 6
CD34	Cluster of differentiation 34
CD31	Cluster of differentiation 31
Cpm	Counts per minute
DAB	3,3'-diaminobenzidine
DAPI	42,6-diamidino-2-phenylindole, dihydrochloride
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ds	Double stranded
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
ETOH	Ethanol
GAD	Glutamic acid decarboxylase
HCl	Hydrochloric acid
HGF	Hepatocyte Growth Factor
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell
IL-1 β	Interlukin-1beta
IBMIR	Instant blood mediated inflammatory response
IFN- γ	Interferon gamma
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay

FACs	Fluorescence activated cell sorting
FCS	Foetal calf serum
FGF	Fibroblast Growth Factor
HbA1c	Glycated haemoglobin
HLA	Human Leukocyte Antigen
IA2	Islet tyrosine phosphatase
IEC	Islet endothelial cell
IFN- γ	Interferron-gamma
IL-1 β	Interleukin -1 beta
i.p.	Intraperitoneal
IPGTT	Intraperitoneal glucose tolerance test
MMP	Matrix metalloprotease
MCP	Monocyte chemoattractant protein
MEM	Modified Eagle's Medium
MMP	Matrix Metalloproteinase
MHC	Major Histocompatibility Complex
MSC	Mesenchymal Stem Cell
NF- κ B	Nuclear factor kappa B
PDX-1	Pancreatic duodenal homeobox-1
PECAM	Platelet endothelial cell adhesion molecule
PPI	Pre Proinsulin
PBS	Phosphate Buffered Saline
RIA	Radioimmunoassay
RM	Repeated measurements
STZ	Streptozotocin
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TF	Tissue factor
TGF- β	Transforming growth factor beta
TNF- α	Tumour necrosis factor-alpha
TBS	Tris-Buffered Saline
VEGF	Vascular Endothelial Growth Factor
vWF	von Willebrand Factor

1 Chapter One - General Introduction

1.1 Diabetes Mellitus

Diabetes mellitus is a chronic endocrine disorder which occurs when the body cannot produce enough insulin or does not use the insulin that it is able to produce effectively (World Health Organisation (WHO)). Diabetes mellitus is separated into three major subgroups, as recognised by the WHO; Type 1 Diabetes Mellitus (T1DM), formally known as Insulin Dependent Diabetes Mellitus (IDDM), Type 2 Diabetes Mellitus (T2DM) or Non-Insulin Dependent Diabetes Mellitus (NIDDM) and gestational diabetes, which is first recognised during pregnancy and is temporary in the majority of cases. All forms of the disease are characterised by hyperglycaemia (elevated blood glucose concentrations), due to defects in insulin secretion, insulin action on peripheral target tissues, or both, but their aetiology and therefore treatment are quite distinct. Hyperglycaemia is associated with a number of symptoms including excessive weight loss, polydipsia (extreme thirst), polyuria (excessive urine production) and blurred vision, which are generally present at the time of clinical diagnosis. In healthy individuals blood glucose concentrations are maintained between 3-7mmol/l, thus patients with diabetes mellitus must be given treatment to ensure that glycaemia remains as close to the normal physiological range as is possible. Unfortunately, this can be difficult and suboptimal glucose homeostasis can lead to a number of chronic microvascular complications, including nephropathy, neuropathy, retinopathy and limb amputations.

Recent figures indicate that the number of people living with diabetes worldwide is expected to rise from 366 million in 2011 to 552 million by 2030 (International Diabetes Federation, Diabetes Atlas, 5th Edition). T2DM is by far the most common form of diabetes (approximately 80 percent of cases), which is usually associated with obesity and therefore an inability of the insulin producing β -cells to match the demand for insulin. Ultimately, obesity can cause insulin resistance of peripheral tissues, such as the liver, muscle and adipose tissue (van Belle et al., 2011), which eventually leads to β -cell failure. T1DM accounts for approximately ten percent of cases and has a

complex aetiology resulting in the autoimmune attack of the pancreatic β -cells, eventually leading to a complete lack of endogenous insulin production (see section 1.2). The increased incidence of T2DM is increasing most notably, largely due to an increase in obesity, however the incidence of T1DM is also rising (van Belle et al., 2011). Alarming, the greatest increase in T1DM is that seen in children under 5 (Patterson et al., 2009), with no known way of preventing this pandemic (Todd, 2010). The increasing incidence of diabetes mellitus and associated long-term complications places significant financial burden upon national healthcare services and countries, with approximately 11 percent of healthcare expenditures in the world due to diabetes (International Diabetes Federation, Diabetes Atlas, 5th Edition).

1.2 Aetiology of Type 1 Diabetes

T1DM results from the autoimmune destruction of the insulin secreting pancreatic β -cells (Atkinson and Eisenbarth, 2001). It is generally accepted that T1DM has a T-cell mediated pathology; macrophages and T-cells invade the islets in an inflammatory reaction termed insulinitis, with CD4⁺ T-cells enhancing the ability of CD8⁺ T-cells to kill the islet β -cells (Skowera et al., 2008), expressing auto-antigenic peptides, such as insulin, glutamic acid decarboxylase (GAD) and islet tyrosine phosphatase (IA-2) (Palmer et al., 1983, Baekkeskov et al., 1990, Payton et al., 1995). Disease progression and development of overt diabetes often takes a number of months to years, over which the loss of β -cells exceeds the rate at which they are replaced (Atkinson and Gianani, 2009). Clinical symptoms do not arise until 70-80 percent of the β -cells have been destroyed (Kloppel et al., 1985), which results in insufficient endogenous insulin and the need for exogenous insulin therapy to maintain blood glucose homeostasis (Zimmet et al., 2001). The aetiology is complex, involving genetic susceptibility to the disease, but also environmental factors (Atkinson and Eisenbarth, 2001). Disease concordance in monozygotic twins is approximately 50 percent, suggesting a strong genetic component (Redondo et al., 2008). A number of susceptibility genotypes predispose individuals to the autopathogenic response mounted by their own immune system. However, a number of genotypes and environmental factors have also been shown to be protective. Under conditions in which the protective genotypes and environmental factors are not sufficient to overcome the functions of the susceptibility genotypes, the autoimmune response progresses until the development of overt

diabetes when clinical symptoms are evident. Predisposing variants of T1DM genes of most importance include the human leukocyte antigen (HLA) genes (Risch, 1987), which confers the greatest risk for T1DM, as well as variants of the insulin (INS) gene (Bell et al., 1984, Lucassen et al., 1993). HLAs are a family of homologous proteins, which present antigenic peptides to both effector- and regulatory- T-cells (Polychronakos and Li, 2011). The ultimate fate of the β -cell depends upon the functions and relative contributions of these T-cell subsets, with evidence to suggest that effector T-cells from patients with T1DM have a reduced capacity to be suppressed by regulatory T-cells (Lawson et al., 2008). This raises the interesting possibility that targeting regulatory T cells, to enhance their immunosuppressive functions may help to enhance β -cell specific tolerance in pre-diabetic patients (Tree et al., 2010), providing a potential mechanism for delaying or preventing the development of overt diabetes. Without interventional immunotherapy, dysregulation of the innate immune system may trigger multiple aspects of the adaptive immune system to be altered resulting in the eventual loss of immune self-tolerance to β -cell antigens. A number of peptides from these β -cell antigens have now been identified, as reviewed by Lorenzo and colleagues (Di Lorenzo et al., 2007). This information may enhance the current understanding of the role of auto reactive T-cells in disease pathology and aid the design of antigen-specific immunotherapy (Raz et al., 2001).

The rising incidence of T1DM is too rapid to be fully attributed to genetic components, thus it seems that several changes in lifestyle are likely to be accelerating the disease progression, leading to a younger age of diagnosis in many patients (Patterson et al., 2009), as well as increasing the incidence in all age groups. Changes in diet, physical activity patterns, body-size, early life infectious disease patterns, climate, pharmaceuticals and vaccination rates are all potential influences (Vehik and Dabelea, 2011). Environmental triggers, such as viral infections are also likely to contribute to the pathogenesis (Hyoty and Taylor, 2002, Richardson et al., 2009, Stene et al., 2010), however no exogenous factor or infectious trigger is actually required (Todd, 2010). The geographical distribution of T1DM may also provide information regarding the aetiology of diabetes, with differences in both genetic and environmental components likely to contribute to the higher incidence of diabetes in northern European countries compared to those on the Mediterranean (Borchers et al.,

2010). Much of the geographical variation in the incidence of T1DM is thought to be due to different distributions of ethnicity/race worldwide, with Caucasians being at greater risk than all other race/ethnic groups. At present, exogenous insulin therapy is the main treatment for patients with T1DM, although continued research efforts into improving allogeneic islet transplantation may potentially enable the more widespread application of this as a treatment for the disease (see section 1.4 for a more detailed description of treatment options).

1.3 Islets of Langerhans

The pancreas is divided into two major components. The majority of the pancreas (approximately 98 percent) is exocrine tissue, which serve as the major source of digestive enzymes in the body and ensures the breakdown of lipids, proteins and polysaccharides. The endocrine component of the pancreas consists of small clusters of cells called islets of Langerhans; first described by Paul Langerhans in 1869. These highly vascularised endocrine mini-organs are of limited size and are dispersed throughout the exocrine pancreas, as opposed to being merged into a single solid organ. The human pancreas contains 1-2 million islets of Langerhans, which contribute 1–2 percent of the gland (Pipeleers et al., 1994). Each islet ranges from approximately 50-400 μ m in diameter, regardless of the species studies, with each islet consisting of 1,000-3,000 endocrine cells (Feldman, 1979). Approximately 65–80 percent of the endocrine cells in rodent islets are insulin secreting β -cells, 15-20 percent glucagon secreting α -cells, 3-10 percent somatostatin secreting δ -cells and 3-5 percent pancreatic polypeptide secreting PP cells (Elayat et al., 1995). There are differences in islet architecture between species, with rodent islets typically consisting of a central core of β -cells, surrounded by α -, δ - and PP cells in the islet mantle (Kim et al., 2009). This arrangement of endocrine cells favours homologous interactions between β -cells, which are favourable for normal rodent islet function (Bergsten et al., 1994, Hauge-Evans et al., 1999, King et al., 2007). In particular, homologous cell interactions increase insulin gene expression, insulin content and glucose stimulated insulin secretion (Bosco et al., 1989, Wojtusciszyn et al., 2008). In contrast, the distribution of endocrine cells in human islets is more heterogeneous (Cabrera et al., 2006), which means that the numbers of homologous β -cell contacts are reduced compared with rodent islets (Wojtusciszyn et al., 2008). The physical proximity of α -,

δ - and β -cells in human islets ensures the tight co-ordination of blood glucose homeostasis, as the α - and δ -cells are distributed throughout the islet. In rodent islets, only a small number of α - and δ -cells are juxtaposed with the β -cells forming the islet core, however it seems that the microcirculatory system allows for the co-ordinated responses to fluctuations in blood glucose (Unger and Orci, 2010). For both rodent and human islets, β -cells have heterogeneous insulin secretory activity, with only a small proportion of β -cells in each islet contributing to the majority of insulin secreted (Hiriart and Ramirez-Medele, 1991, Bosco et al., 1995, Wojtuszczyk et al., 2008). Interspersed amongst the endocrine cells are nerves, fibroblasts, macrophages, dendritic cells and endothelial cells (ECs).

There is a complex interplay between the endocrine cells within the islets, which ensures the fine-tuning of blood glucose homeostasis, maintaining blood glucose concentrations between 3 and 7mmol/l in healthy individuals. The pancreatic β -cells are the only cell type in the body which both synthesise and secrete the 6kDa polypeptide hormone insulin. Insulin stimulates uptake of glucose from the blood stream via GLUT-4 glucose transporters, expressed on adipose and skeletal muscle tissue and GLUT-2 glucose transporters expressed on liver tissue. Insulin also activates glycolysis and glycogen and fat synthesis (glycogenesis and lipogenesis) and simultaneously inhibits the breakdown of glycogen (glycogenolysis). Glucokinase serves the glucose sensor in pancreatic β -cells (Matschinsky, 1996), ensuring that they can rapidly respond to fluctuations in blood glucose concentration, maintaining normoglycaemia accordingly. Increased plasma glucose concentration is the primary stimulus for insulin secretion, however, a number of other nutrient and non-nutrient stimuli, such as the amino acids arginine, leucine and lysine, are involved in the regulation of insulin secretion. Insulin is also thought to exert an autocrine effect through its actions on insulin receptors expressed on β -cells, helping to protect β -cells from apoptosis and increasing pro-insulin gene expression, as well as β -cell proliferation (Persaud et al., 2008).

Glucagon is a catabolic hormone secreted by α -cells, which increases blood glucose concentrations, mainly through its actions on the liver, promoting glycogenolysis and gluconeogenesis. This is important physiologically as it helps to protect healthy

individuals from hypoglycaemia. Somatostatin is secreted by the δ -cells, exerting a tonic inhibitory effect on both β - and α - cells to prevent the secretion of insulin and glucagon respectively (Kanno et al., 2002, Hauge-Evans et al., 2009). This helps to fine-tune blood glucose regulation.

The specific organisation of cells within the islet suggests a coupling between morphology and function, with two major mechanisms by which both endocrine and non-endocrine cells within the islets communicate with each other. The first is via paracrine interactions, in which a secretory product from one cell moves a short distance through the interstitial fluid to reach a target cell. The second mechanism is via the islet vascular system, with the co-ordinated function of all islet cell types ensuring optimal blood glucose homeostasis (Weir and Bonner-Weir, 1990).

1.3.1 Islet microvasculature

The islet microvasculature has been described as glomerular-like since its initial description by Paul Langerhans in 1869. The pancreatic β -cells are closely associated with the islet microvasculature, with the developmental process ensuring that every β -cell is no more than one cell away from a capillary (Lammert et al., 2001). The close association between β -cells and islet endothelial cells (IECs), ensures that β -cells are able to respond rapidly to fluctuations in blood glucose concentration; secreting insulin into the circulation and maintaining tight blood glucose homeostasis (Lammert et al., 2001). The order in which the islet endocrine cells are perfused is debatable, but studies have indicated that the predominant pattern of blood flow is, 'inner-outer', with the β -cells being perfused before the α - and δ -cells (Samols et al., 1988, Stagner and Samols, 1992, Menger et al., 1994, Nyman et al., 2008). The blood perfusion of endogenous pancreatic islets is highly variable and tightly regulated by blood glucose, but is approximately 10x higher than that of the surrounding exocrine pancreas (Lifson et al., 1980, Lifson et al., 1985), despite only constituting 2 percent of the gland. Additionally, the oxygen tension of the islets is higher than the surrounding exocrine pancreas (31-37mmHg and 20-23mmHg, (Carlsson et al., 1998)), although emerging evidence suggests that approximately 25 percent of all islets show low oxygenation (<10mmHg), reflecting a subpopulation of functionally dormant islets (Olsson and Carlsson, 2011). The vascular density of the endocrine pancreas is higher

than that of the surrounding exocrine pancreas, with wider more tortuous vessels present (Brissova et al., 2006). Furthermore, IECs have 10x as many fenestrae as the ECs in the exocrine pancreas (Henderson and Moss, 1985), which allows for the rapid trans-endothelial transport of insulin into the circulation (Lammert et al., 2001). This high vascular density is important for the adequate provision of oxygen and nutrients, as well as continuous glucose sensing and dispersion of hormones to target tissues. The dense vascularisation of the islets is also important for β -cell replication, which is particularly crucial at times of increased demand for insulin, such as during pregnancy or obesity (Johansson et al., 2006b, Nikolova et al., 2006).

1.3.2 Intraislet Endothelial Cells

ECs line all blood vessels forming a continuous monolayer layer between the blood and interstitial fluid. ECs are typically elongated cells, which are approximately 30 μ m long, 12 μ m wide and 0.3 μ m deep. They are adapted to meet the individual needs of each organ and are therefore both phenotypically and functionally heterogeneous (Carmeliet, 2003), with differences observed between species, as well as different sized vessels and different organs (Hewett and Murray, 1993, Cines et al., 1998). All ECs function as an immunological and physical barrier between the blood and tissues and play an important role in angiogenesis (Cines et al., 1998). Increasing evidence suggests that IECs do not function solely as a transport system, but that their close proximity to β -cells ensures that they are exposed to each other's products and hence a number of EC-derived molecules including laminins (Johansson et al., 2009a), Hepatocyte Growth Factor (HGF) (Johansson et al., 2006b), thrombospondin-1 (Olerud et al., 2011) and endothelin-1 (Gregersen et al., 1996) have been shown to be important for β -cell function (as represented in Figure 1.1). Furthermore, it is thought that β -cells rely on adjacent ECs to form a basement membrane (Nikolova et al., 2007), consisting largely of laminins, collagen IV, and fibronectin, which are also associated with enhanced β -cell function and survival (Perfetti et al., 1996, Nagata et al., 2001, Kaido et al., 2006, Jalili et al., 2011). EC-derived products have been shown to improve β -cell function through enhanced glucose stimulated insulin secretion, islet insulin content and glucose oxidation rate (Johansson et al., 2009a). ECs also produce vasoconstrictors and vasodilators, which are important for regulating islet blood flow. β -cell secreted VEGF-A generates a permeable endothelium allowing insulin to be

secreted rapidly into the bloodstream and is also responsible for the large number of ECs within the islets (Lammert et al., 2003). There is also evidence to suggest that vascular supporting cells, such as pericytes play an important role in β -cell homeostasis (Clee et al., 2006).

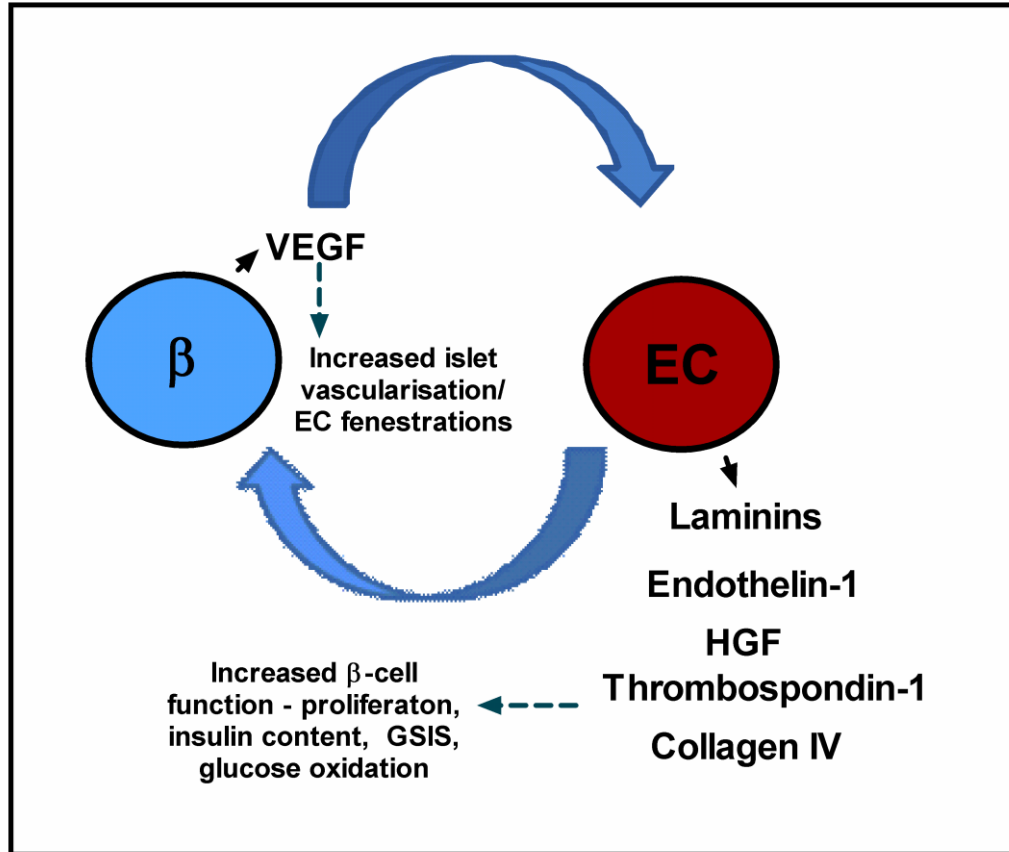


Figure 1.1 Schematic representation of cross-talk relationship between β -cells and islet ECs. A number of paracrine interactions take place between β -cells and islet ECs, enhancing islet function and survival.

As well as being phenotypically heterogeneous, ECs also show tremendous phenotypic plasticity (Carmeliet, 2003), when subjected to different microenvironments both *in vitro* and *in vivo*. This plasticity can be seen in the revascularisation of islet grafts, where ECs from the host's implantation organ, which contribute to the newly formed islet vasculature, appear to obtain the phenotypic characteristics typical of intraislet ECs regardless of the implantation organ (Carlsson et al., 2001). IECs express typical cell adhesion molecules, such as V-CAM 1, PECAM (CD31), VE-cadherin, von Willebrand factor, thrombomodulin, CD34, ICAM-1 and endoglin (CD105), as well as more specialized molecules, such as the platelet activating factor (PAF) receptor nephrin (Zanone et al., 2005). However, it has also

been shown that 84 percent of the receptors expressed on IECs are unique to the islet vasculature and cannot be found in the surrounding exocrine tissue (Yao et al., 2005).

1.3.3 Endothelial Cell surface markers

Various techniques have previously been used to quantify IECs. These include the use of antibodies, lectins and also di-acetylated low-density lipoproteins. Some techniques used are suitable for human ECs, but not rodent ECs and it is therefore important to choose an appropriate method for assessing vascular density in endogenous, freshly isolated and cultured islets, as well as transplanted islets. Furthermore, certain markers do not work when used on formalin fixed, paraffin embedded sections and require an alternative fixation or embedding method (Mattsson et al., 2002a). There are certain difficulties in staining appropriately for IECs, due to the vast heterogeneity of ECs. Mattsson et al addressed this problem and analyzed the staining properties of several different EC markers used for immunohistochemistry of formalin fixed, paraffin embedded sections. These included CD34, CD31, CD200, O_x43, von Willebrand factor and the lectins *Ulex europaeus* and *Bandeiraea simplicifolia* (BS-1) (Mattsson et al., 2002a). It was shown that CD34 does not stain ECs in all organs tested in mice and rats, but does in the kidney, pancreas and small intestine of C57Bl/6 mice. Although, this observation suggests that CD34 is not a marker that can be used to assess vascular density in all organs, it was shown to be a reliable marker for IECs in the native pancreas, isolated islets and islets transplanted beneath the kidney capsule. BS-1 was also shown to stain pancreas and kidney sections positively for ECs, whereas, CD31 and vWF did not stain pancreas sections. This study shows that both CD34 and BS-1 are suitable markers for identifying ECs in native, isolated and transplanted mouse islets. It is however recognized that other EC markers would also be appropriate under different experimental conditions, such as for the analysis of cryosectioned tissues. Additionally, the same marker from different manufacturing companies may also work more or less well in a specific setting. Discrepancies within the literature concerning results obtained for various markers may also involve methodological factors, such as whether or not an antigen retrieval step is used, which affects whether or not certain markers are detected.

1.3.3.1 CD34 as an Endothelial Cell marker

Antibodies against CD34 have been recognized to bind ECs in mice (Baumhueter et al., 1994, Garlanda et al., 1997, Dath et al., 2011) and humans (Fina et al., 1990, Muller et al., 2002b, Dath et al., 2011). More specifically, they have also been shown to bind to islet ECs in both mice (Mattsson et al., 2002a) and humans (Bosco et al., 2010). The CD34 rat monoclonal antibody binds to the CD34 cell surface antigen on capillary ECs. This antigen is a transmembrane glycoprotein of 115kDa, which is expressed on hemopoietic progenitor cells as well as the vascular endothelium (Fina et al., 1990). The fact that CD34 is expressed on hemopoietic progenitor cells does not lead to false positive identification of ECs, as haematopoiesis in the adult is a process that takes place in the bone marrow, lymphatic organs and the liver. Therefore, hemopoietic progenitor cells are unlikely to be detected in significant numbers within islets. It has previously been shown that capillaries in most tissues express CD34 mRNA and stain positively when using all seven of a set of CD34 monoclonal antibodies. However, in contrast to small blood capillaries, as are present within islets, it was also shown a lot of the larger blood vessels are not CD34+ (Fina et al., 1990).

Functionally, CD34 is thought to be involved in cell interactions and cell adhesion, playing an important role in leukocyte trafficking (Baumhueter et al., 1994, Muller et al., 2002b). The CD34 gene belongs to a cluster of genes on chromosome 1q encoding adhesion molecules (Stella et al., 1995), in support of this hypothesis. The highly negative charge of the fully glycosylated CD34 protein, means that CD34 is unlikely to mediate homotypic intracellular adhesion between ECs on its own (Fina et al., 1990), which is supported by the observation that CD31 molecules are expressed on EC membranes, with CD31 also functioning as a cell adhesion molecule (see section 1.3.3.2).

The phenotype of ECs is unstable and particularly likely to change during culture, where the native microenvironment of the EC is altered. It has been shown that ECs can lose their tissue specific traits *in vitro* (de Bono and Green, 1983). In particular, ECs have been shown to lose their fenestrations during culture (Borsum et al., 1982, Milici et al., 1985). Specifically, with regards to CD34 expression; Fina et al have

shown that the CD34 antigen is not detectable on the cell surface of ECs cultured under a variety of conditions promoting proliferation or differentiation. However, the CD34 mRNA is detectable suggesting that CD34 may be down regulated or processed into a form not recognizable by CD34 antibodies during culture (Fina et al., 1990). Studies have indicated that CD34 can be regulated by cell contact or changes in the extracellular microenvironment (Korff and Augustin, 1998). Other studies have shown that CD34 expression may be down regulated during inflammatory cytokine exposure (Norton et al., 1993, Delia et al., 1993); however this suggestion is not supported by all studies (Baumhueter et al., 1994). Other evidence suggests that CD34 molecules are expressed at the tip of vascular sprouts and that CD34 is upregulated during angiogenesis (Schlingemann et al., 1990, Miettinen et al., 1994). Discrepancies in the literature regarding the exact conditions, under which CD34 is expressed, particularly *in vitro*, emphasize the benefits of using more than one EC marker for analysis of islet vascular density.

1.3.3.2 CD31 as an Endothelial Cell marker

CD31, otherwise known as Platelet/Endothelial Cell Adhesion molecule-1 (PECAM 1) is a 130kDa integral membrane glycoprotein localised to the intercellular borders of ECs. CD31 functions as a cell adhesion molecule (Albelda et al., 1991, Muller et al., 2002a) and had been suggested to play a role in forming or stabilizing the vascular bed. CD31 is also found on the surface of platelets, monocytes, neutrophils and selected T-cell subsets, but it is far less abundant here than on ECs (Muller et al., 2002a). CD31 may play a role in leukocyte motility (Newman, 1994) and is thought to be important for mediating homophilic interactions between ECs during angiogenesis (Sun et al., 1998, Nakada et al., 2000). In general, CD31 is considered to be constitutively expressed on all ECs and is therefore classified as a pan endothelial cell marker (Scholz and Schaper, 1997). However, this view of an average or 'generic' EC has been doubted by others (Danilov et al., 2001). Furthermore, as with CD34, some studies have shown that CD31 expression can be down regulated by the inflammatory cytokines TNF- α and IFN- γ (Stewart et al., 1996). Again, the heterogeneity of ECs and their capacity be regulated by the surrounding microenvironment, emphasizes the benefits of using multiple EC markers to investigate EC density and distribution.

1.3.3.3 *Bandeiraea simplicifolia* as an Endothelial Cell marker

Lectins are proteins, which are usually derived from plants. *Bandeiraea simplicifolia* (BS-1) is a lectin which has been shown to be a selective and versatile marker for the islet endothelium. It is a tetrameric protein with a high affinity for terminal α -D-galactosyl residues, which are known to be present on all ECs. BS-1 and its isoform *Griffonia simplicifolia* both bind to α -D-galactosyl residues, suggesting that ECs have a highly specific glycosylation pattern (Laitinen, 1987). This selectivity may explain why Mattsson and colleagues found that BS-1 consistently stained IECs regardless of the species and tissue studied (Mattsson et al., 2002a). However, BS-1 has also been shown to give diffuse background staining of pancreatic acinar cells and also stains epithelial cells (Mattsson et al., 2002a).

1.4 Treatment of Type 1 Diabetes Mellitus

For most patients with T1DM, the therapy of choice is that of daily subcutaneous insulin injections (Vardanyan et al., 2010). The discovery of insulin in the 1920's (Banting and Best) has meant that patients with T1DM are able to lead a near normal lifestyle, with reasonable blood glucose control. Insulin therapy is effective at treating symptoms, which include polyuria, ketoacidosis, hyperglycaemia, tiredness, weight loss, polydipsia and blurred vision. Under normal circumstances, insulin is released in a pulsatile manner, resulting in small and frequent oscillations in plasma insulin concentrations (Goodner et al., 1977), which ensure tight regulation of blood glucose homeostasis. Subcutaneous insulin injections cannot provide the fine tuning of blood glucose control that occurs under normal physiological conditions and thus fluctuations in blood glucose concentration mean that insulin therapy does not eliminate the risk of chronic secondary complications that can occur with T1DM, despite reasonable glycaemic control in some patients (Nathan et al., 2005). In particular, these complications include neuropathy, nephropathy, retinopathy, limb amputations and cardiovascular disease, which account for most of the morbidity and mortality in T1DM (Fiorina et al., 2008). A number of research strategies have focused on methods to improve pharmacological preparations of long-acting insulin, in order to better mimic the physiological situation, where insulin is released from the pancreas into the portal bloodstream at an almost constant rate. Clearly, portal infusion of insulin is not feasible, but efforts to improve the pharmacokinetics and

pharmacodynamics of long-acting insulin analogues have helped to improve metabolic control in many patients. These newer preparations can be taken twice daily, together with a rapid-acting insulin analogue taken at meal times (mimicking the ‘bolus’ component of endogenous insulin secretion in response to nutrients or other challenges), which means that the goal of reducing glycated haemoglobin (HbA1c) levels to less than seven percent with minimal hypoglycaemia, has become a more realistic target (Bolli et al., 2011).

Alternative strategies to optimize blood glucose control have involved the development of insulin pumps, which provides a continuous infusion of insulin into the subcutaneous tissue and has now become the ‘gold standard’ for basal insulin replacement. An advantage of insulin pumps over conventional insulin injections is that the rate at which basal insulin is delivered can be altered, thus allowing for a more regulated delivery of insulin over 24hr (Bolli et al., 2011). Current pumps use an open-loop system, in which the rate of insulin delivery is not automatically adjusted according to blood glucose (Weintrob et al., 2004). However, insulin pumps offer potential future benefits (Alsaleh et al., 2010) of a closed-loop system in which the insulin pump would be linked to a glucose sensor, thereby acting as an artificial pancreas (Weintrob et al., 2004).

Although it is clear that intensive insulin therapy has improved the treatment of T1DM in terms of patient quality of life, metabolic control and risk of long-term complications, it can be associated with an increased risk of hypoglycaemia, which is potentially dangerous or life-threatening. For a small-subset of patients with T1DM (~10 percent) blood glucose control is extremely difficult, due to hypoglycaemia unawareness or erratic blood glucose concentrations leading to repeated and unpredictable hypoglycaemia or uncontrolled hyperglycaemia resulting in ketoacidosis (‘brittle diabetes’). For these patients β -cell replacement strategies by either whole organ pancreas or allogeneic islet transplantation may be the best treatment for ensuring safe and optimal diabetes management (Ludwig et al., 2010). The first whole organ pancreas transplantation was performed more than forty years ago (Kelly et al., 1967), with the majority of pancreas transplants today being performed as multiple organ transplants; usually simultaneous pancreas-kidney

procedures. Until more recently, pancreas transplantation was associated with a higher success rate (in terms of maintained insulin independence) than allogeneic islet transplantation, but there is now evidence to suggest that the two strategies can result in comparable glycaemic control in patients (Maffi et al., 2011), emphasising the vast improvements that have already occurred within the islet transplantation field. An important benefit of allogeneic islet transplantation is that it is less invasive than pancreas transplantation. Therefore, islet transplantation is associated with lower morbidity and a reduced likelihood of surgical complications (Vardanyan et al., 2010, Ludwig et al., 2010, Maffi et al., 2011). Notably, the high surgical risk with pancreas transplantation means that it is not a viable option for patients with cardiovascular risks, whereas islet transplantation may be. Additional benefits of islet transplantation include the option for repeated islet infusions if required. Direct comparison of these β -cell replacement strategies is however difficult and the choice of islet or pancreas transplantation is complex, requiring an important consideration of the patients metabolic complications, pre-existence of diabetic complications and psychological situation of the patient and family (Ludwig et al., 2010).

1.4.1 Clinical islet transplantation

Successful islet transplantations were initially performed in rodents (Ballinger and Lacy, 1972) and then for the first time in humans in 1990 (Scharp et al., 1990). Although this meant that allogeneic islet transplantation emerged as a means to restore insulin secretory capacity in patients with T1DM, the overall success rate in terms of insulin independence was low (approximately 10 percent). It was not until the establishment of the landmark Edmonton protocol in 2000 (Shapiro et al., 2000) that islet transplantation became a serious option for patients with T1DM. The enthusiasm evoked from this study was due to the 100 percent rate of insulin independence achieved during the first year following islet transplantation to seven patients with a history of severe hypoglycaemia in Edmonton, Canada. To achieve this, the Edmonton protocol requires that a large number of islet cells, involving multiple islet infusions, from more than one brain-dead donor are transplanted (> 9,000 islet equivalents per kg body weight). Additionally, the protocol includes the use of a specific glucocorticoid-free immunosuppressive regimen and transplantation of fresh, as opposed to cultured islets (Shapiro et al., 2000). Although transplantation

using the Edmonton protocol drastically improved the rate of insulin independence, compared with previous clinical islet transplantations, the longer term outcomes in terms of graft failure and a return to the need for exogenous insulin therapy were highly disappointing, with less than ten percent of patients remaining insulin independent after five years. In a follow-up study using a larger cohort of 65 patients, similar transplantation outcomes were achieved with 69 percent of recipients remaining insulin independent at one year and only 7.5 percent at five years (Ryan et al., 2005a). More recently, 57 percent of patients were shown to be insulin independent at three years after receiving islet transplantation with the Edmonton protocol (Vantyghem et al., 2009). A number of islet transplant centres worldwide have demonstrated the reproducibility of the Edmonton protocol (Alejandro et al., 2008), but also emphasise a number of problems which must be addressed to enable the more widespread application of clinical allogeneic islet transplantation to the majority of patients with T1DM.

Longer term follow-up of transplant recipients has also meant that experts in the field have changed the way in which they define successful islet transplantation. Initially, successful islet transplantation was measured by the ability of the graft to maintain glycaemic control, with the patient being free from the need for exogenous insulin therapy ('insulin independence') during the first year. Secondary endpoints included improved HbA1c levels, basal and stimulated blood C-peptide levels in response to an arginine challenge, as well as insulin independence during the longer term follow-up and reduced mean amplitude of glycaemic excursions (Shapiro et al., 2006). Increasingly, successful transplantation outcome is measured through blood glucose stabilisation; with reduced hypoglycaemia unawareness and frequency of severe hypoglycaemic episodes, as well as reduced vascular complications and the associated improvements in quality of life (Poggioli et al., 2006, Toso et al., 2007, Alejandro et al., 2008, Tharavanij et al., 2008, Robertson, 2010, Speight et al., 2010, de Kort et al., 2011). Despite the frequently observed decline in islet graft function, partial graft function means that only small doses of exogenous insulin are required and this has been shown to confer stabilisation of diabetic complications, including diabetic retinopathy and nephropathy (Fiorina et al., 2003), thus in this sense the islet transplantation can still be viewed as successful.

Some centres have more recently reported insulin independence with single donor transplantation (Hering et al., 2005, Matsumoto, 2011, Posselt et al., 2010). However this is not reproducible in all islet transplant centres and longer term follow-up studies are needed to determine whether single donor transplantation is feasible for longer term insulin independence. The routine achievement of single-donor islet transplantation success is important for increasing the number of patients who can be given allogeneic islet transplants, but also for increasing the acceptability of allogeneic islet transplantation within the transplant community, given that whole organ pancreas transplantation requires only a single donor (Shapiro, 2011b). Recent strategies have focused on reducing the number of islet donors per recipient by increasing the functional β -cell mass before, during and after transplantation. Attempts to do so have largely focused on enhancing islet engraftment, which is currently suboptimal and therefore results in substantially reduced islet function and cell death during the post transplantation period (detailed in section 1.5). Strategies include improvements in the microenvironment to which the islets are transplanted and in particular; enhancing islet graft revascularisation for improved oxygenation. Additionally, substantial efforts to reduce inflammation and improve immunosuppressive regimens, or indeed prevent the need for immunosuppressive drugs at all, are also being made to improve donor: recipient ratios. At present, inadequate islet function and survival during the post transplantation period, taken together with the scarcity of donor islet material and significant immunological barriers, means that islet transplantation is currently limited to patients with recurrent hypoglycaemia, hypoglycaemia unawareness or T1DM after a kidney transplant (de Kort et al., 2011). Thus, efforts to overcome these problems are clearly warranted.

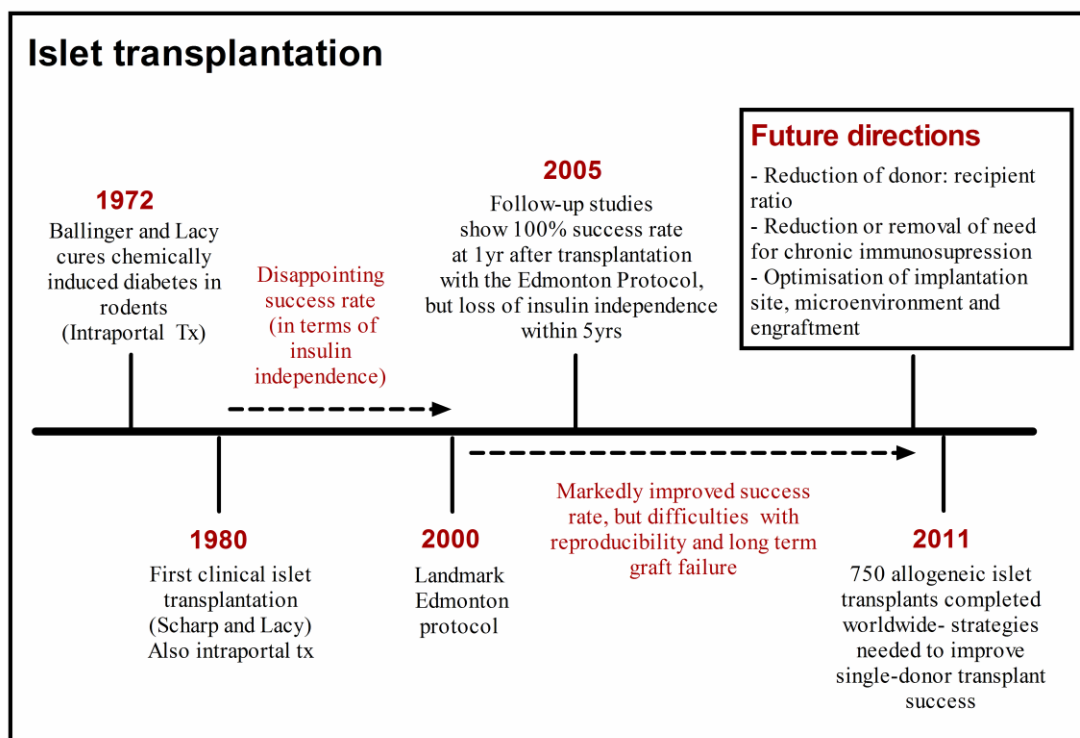


Figure 1.2 Time-line for islet transplantation. Substantial improvements have already been made within the islet transplantation field. However, a number of problems associated with graft rejection and inadequate engraftment prevent the widespread application of allogeneic islet transplantation.

Significant challenges to the islet transplantation field are associated with substantial islet loss due to allo- and autoimmunity, which contributes to the decline in graft function and loss of insulin independence (Harlan et al., 2009). Induction of immunological tolerance would reduce or prevent the need for life-long immunosuppression and is one of the major goals towards enabling the widespread application of islet transplantation (Ricordi and Strom, 2004, Shapiro, 2011a). Despite the fact that immunosuppressive therapies have improved dramatically, with the development of the Edmonton protocol, which introduced a steroid-free regimen (Shapiro et al., 2000), as well as significant advances since this (Bertuzzi et al., 2002, Ricordi, 2003, Roelen et al., 2009, Posselt et al., 2010), transplant recipients still need to live with life-long immunosuppression. This is associated with adverse side effects, which can cause deterioration in quality of life for the patient, which in many cases means that the costs of this out-weigh the benefits of the islet transplant. In particular, side effects include painful mouth ulcers, peripheral oedema and poor wound healing. Additionally immunosuppressive drugs can be nephrotoxic (de Mattos et al., 2000, Froud et al., 2005, Senior et al., 2007, Warnock et al., 2008), which is unacceptable

given that one of the positive outcomes of islet transplantation is the reduction or prevention of diabetic complications including diabetic nephropathy. Furthermore, immunosuppressive drugs can have deleterious effects on the islet cells themselves (Maes et al., 2001, Desai et al., 2003, Shapiro et al., 2005, Nir et al., 2007), which can compromise long term graft function. Recent strategies to improve recipient immunosuppression have included T-cell depletion therapies using an anti-CD52 antibody (Shapiro, 2011a) and T-cell depletion with co-stimulation blockade, using belatacept to block co-stimulation signalling through the CD80-CD86 pathway (Posselt et al., 2010). Other strategies to prevent allogeneic graft rejection include immunoisolation of islets through various encapsulation technologies, such as nanoencapsulation (Pickup et al., 2008). Additional novel approaches to protect the transplanted islets from immune attack included cellular-based strategies, including coating of islets with regulatory T-cells (Marek et al., 2011) or Mesenchymal Stem Cells (MSCs) (Duprez et al., 2011). An extensive review of the current strategies to improve recipient immunosuppression is beyond the scope of this thesis, which is focused primarily on improving transplantation outcome through enhanced morphological and vascular engraftment. However, it is acknowledged that successful transplantation outcome depends not only on replacing the lost β -cells, but also preventing re-current autoimmunity (Aguayo-Mazzucato and Bonner-Weir, 2010). In this context, MSCs provide an ideal cellular candidate for improving allogeneic islet transplantation outcome, as discussed in section 1.6.2.

1.5 Engraftment of transplanted islets

Engraftment is the adaption of the transplanted cells to their implantation organ with regards to revascularisation, reinnervation, and reorganisation of other stromal components (Jansson and Carlsson, 2002) and is essential for the long term survival and function of transplanted islets. For optimal islet function and survival, this process should restore normal islet architecture and morphology; maintaining the typical endocrine cellular composition and restoring the vascular connections, which are disrupted during the isolation, culture and transplantation procedures.

1.5.1 Microenvironment of implantation site

Clinical islet transplantations have almost exclusively been done through intraportal islet infusion using the percutaneous approach, with subsequent embolisation of islets in the portal vein, since the early pioneering work of the late Dr Paul Lacy (Kemp et al., 1973, Ballinger and Lacy, 1972). More recently, experimental and clinical transplantations have suggested that alternative transplantation sites, including the intramuscular site may provide a more favourable microenvironment for the transplanted islets (Rafael et al., 2008, Christoffersson et al., 2010). Intramuscular islet transplantation has shown variable success (Axen and Pi-Sunyer, 1981, Lund et al., 2010), which appears to be related to the technical transplantation procedure. In particular, transplantation of islets as clusters is detrimental for transplantation outcome, due to the development of fibrosis at central parts of the graft (Christoffersson et al., 2010). In contrast, when islets are transplanted in a ‘pearls-on-a-string’ fashion, which ensures that they are engrafted as single islets, transplantation outcomes are more favourable (Lund et al., 2010). This suggests that dispersion of islets at the implantation site, as opposed to transplanting the islets as aggregates, may be important for transplantation outcome. A number of research strategies have focused on alternative implantation sites because of questions regarding detrimental effects that the hepatic intraportal environment can have on the transplanted islets (Lau et al., 2007). In particular, islets transplanted intraportally are subjected to risks associated with gluco- and lipotoxicity (Lee et al., 2007), as well as the toxicity of immunosuppressive drugs that are present in high concentrations in the portal system (Redmon et al., 1996, Shapiro et al., 2005). Poor islet oxygenation due to the low oxygen tension of the liver parenchyma (Carlsson et al., 2000, Carlsson et al., 2001) and inadequate vascular engraftment (see 1.5.2), as well as the instant blood mediated inflammatory response (IBMIR) (Bennet et al., 1999, Moberg et al., 2002) are all important factors driving the search for alternative transplantation sites (Ricordi and Strom, 2004, Robertson, 2004). The kidney subcapsular site is the most commonly used for islet transplantation in rodent studies (Merani et al., 2008), largely due to the ease of retrieving islet grafts for histological analysis and also because the graft-bearing kidney can be explanted to address the issue of endogenous β -cell regeneration. This is particularly important experimentally for dissecting out possible

mechanisms by which a particular modulation to the transplantation procedure might be causing differences in glycaemic control of the recipient animal.

Regardless of implantation site, transplanted islets are subjected to both early and late challenges, which means that a substantial number of islets (as much as 60 percent) are lost through apoptotic or necrotic cell death (Davalli et al., 1996, Biarnes et al., 2002, Miao et al., 2006) and undergo severe changes in islet gene expression, meaning that a large proportion of the remaining islets do not function optimally (Mattsson et al., 2004, Lau et al., 2007, Shi and Taljedal, 1996). Severe functional impairments include reduced glucose oxidation and glucose stimulated insulin secretion and (pro) insulin biosynthesis, as well as reduced mRNA expression of genes essential for β -cell function (Lau et al., 2007). It is important to note that these functional defects are not related to any negative influence that the diabetic milieu would have upon β -cell de-differentiation (Weir et al., 2001), as these studies were carried out in normoglycaemic mice (Lau et al., 2007). This reduction in function contributes to the large number of islets needed to successfully maintain blood glucose homeostasis in both rodents and humans. Although there are indications that transplantation to alternative sites may provide favourable transplantation outcomes (Rafael et al., 2008, Christoffersson et al., 2010), a number of alternative strategies have focused on manipulating the microenvironment to which islets are transplanted in other ways that could potentially be employed at different implantation sites (although this may be affected by the size restrictions of the site). These methods have involved cell co-transplantation strategies, which aim to improve islet cell survival and function by providing trophic support and/or positively influencing the host's immune system (as detailed in section 1.6.2). Other attempts to improve the microenvironment of the implantation site involve tissue engineering approaches, which may also provide trophic support by non-cellular means.

Islets are subjected to a number of cellular stresses resulting from the isolation process, culture period and post transplantation period; all of which contribute to the substantial loss of islet material that is known to occur. During the enzymatic digestion process the islets lose not only their external vascular support, but also their peripheral basement membrane and extracellular matrix (ECM) support (Rosenberg et

al., 1999). Thus, disturbances in the islet microenvironment are responsible for much of the islet loss during the early post transplantation period (Pileggi et al., 2001). Therefore, a number of strategies to improve the microenvironment to which the islets are transplanted have focused on providing the islets with additional ECM support. These strategies often involve the entrapment of islets within ECM scaffolds and several studies have demonstrated an enhancement of both β -cell survival and/or insulin secretory capacity (Perfetti et al., 1996, Nagata et al., 2001, Kaido et al., 2006, Jalili et al., 2011). It is clear that efforts to manipulate the microenvironment of the implantation site or indeed change it both focus heavily on providing appropriate stimuli and conditions for islet revascularisation. This is because it is well established that problems are associated with suboptimal vascular engraftment at a number of sites, which has detrimental effects on islet function and survival, contributing to the decline in graft function and ultimately cell death, frequently observed in both clinical and experimental studies.

1.5.2 Revascularisation of transplanted islets

Two slightly separate mechanisms are involved in embryonic and extraembryonic blood vessel formation: 1) the de novo organisation of ECs into new blood vessels without any contribution from pre-existing blood vessels (vasculogenesis) and; 2) The sprouting of ECs from pre-existing vessels (angiogenesis). Angiogenesis is the predominant mechanism in avascular regions of any tissue (Cines et al., 1998) and is the predominant mechanism of blood vessel formation in the adult (Carmeliet, 2003). The revascularisation process is highly complex, involving the digestion of the vascular wall by proteases and the migration, proliferation and differentiation of ECs (Conway et al., 2001). Furthermore, it is a highly regulated process that occurs in response to tissue demand (Risau, 1997), thus during the early post transplantation period, the islets themselves express angiogenic factors that initiate the process (Vasir et al., 1998). Islets become disconnected from their vascular supply when they are isolated from the pancreas by collagenase digestion. This means that they are avascular during the immediate post transplantation period (Menger et al., 1989, Miao et al., 2006) and therefore need to revascularise. Islet graft revascularisation is important, as the newly formed blood vessels supply the transplanted islets with nutrients and oxygen, thereby dictating the extent to which the β -cells survive

(Carlsson et al., 2000). Thus, the rate at which islets revascularise is important to prevent excessive hypoxia-related cell death (Davalli et al., 1996, Biarnes et al., 2002, Miao et al., 2006), as well as the overall extent of islet revascularisation, which is important for reducing long term graft failure. There is substantial evidence showing that islet graft revascularisation is suboptimal irrespective of the transplantation site used (Mattsson et al., 2002b, Mattsson et al., 2003, Olsson and Carlsson, 2005, Lau and Carlsson, 2009). Islets implanted beneath the renal capsule, spleen or islets transplanted intraportally (human and mouse) all show reduced EC density, which correlates with impaired β -cell function and curative capacity (Lai et al., 2005, Johansson et al., 2009c).

An important consideration regarding islet graft revascularisation is the distribution of ECs at the graft site, in terms of the relative EC density of the endocrine component and the surrounding non-endocrine/stromal component of the graft. It is noteworthy that there are differences regarding the reorganisation of stromal components, between islets transplanted intrapancreatically to their native microenvironment and those transplanted heterotypically (to the liver, spleen or beneath the renal capsule); with a higher percentage of richly vascularised connective tissue present in the grafts of islets transplanted to heterotypic sites (Mattsson et al., 2002b, Mattsson et al., 2003, Olsson and Carlsson, 2005, Lau and Carlsson, 2009). Interestingly in this context, it has been shown that host ECs rarely migrate into the endocrine component of the graft at heterotypic sites (Mattsson et al., 2002b). It is thought that this is likely to be due to a compromised ability of host ECs to migrate into the transplanted islets, because of difficulties associated with islet ECM degradation, but also because the transplanted islets may express high concentrations of angiostatic factors preventing capillary in growth (Johansson et al., 2009b). Differences in the angiogenic response of ECs from different organs/tissues may partially explain these observations; however other factors may also be important. For example, islets transplanted beneath the renal capsule typically fuse to form an aggregated mass of endocrine tissue, which therefore increases the distance over which host ECs must migrate to the centre of the endocrine tissue. Therefore, it is possible that modulation of the microenvironment through alterations in graft morphology may have an important influence on islet revascularisation.

1.5.3 Can islet revascularisation be improved?

Strategies to increase the number of patients who can be treated with the limited human islet material available for clinical islet transplantation encompass ways of improving the survival and function of islet cells via enhanced revascularisation during the post transplantation period and/or enhancing the survival and function of both ECs and β -cells *ex vivo* prior to transplantation, as shown in Figure 1.3.

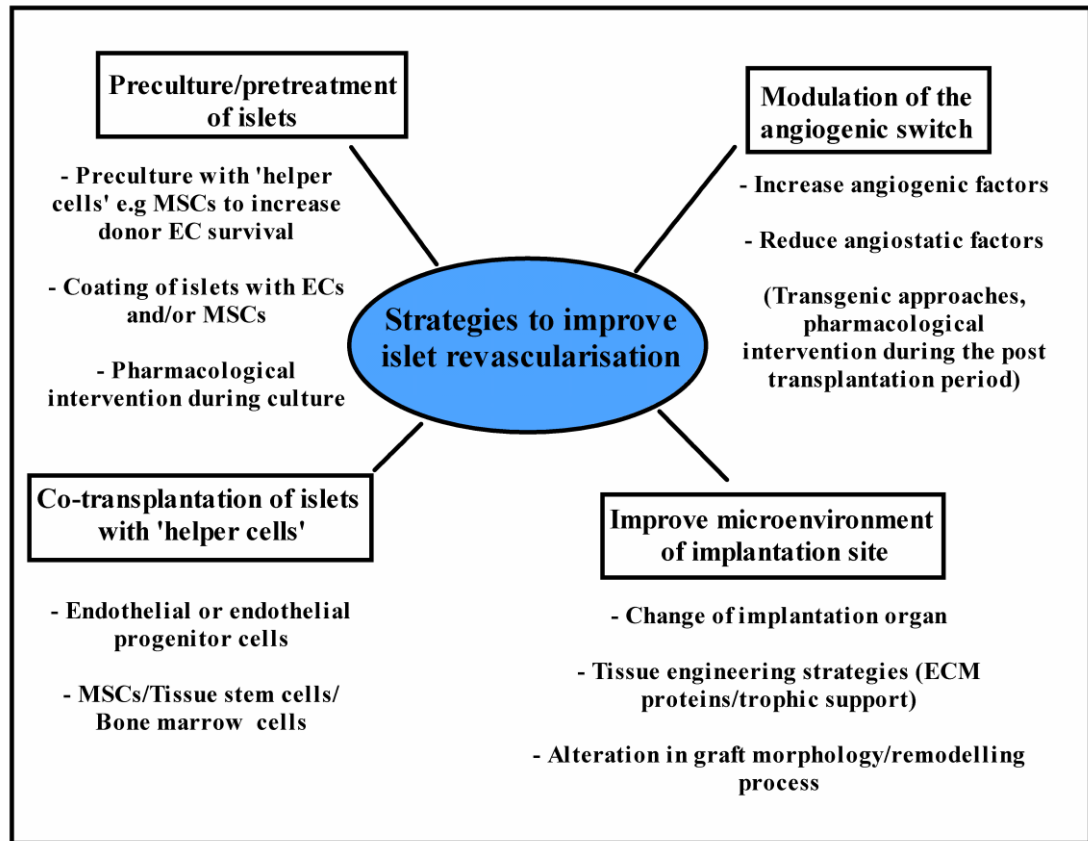


Figure 1.3 Schematic drawing of strategies that have been used to enhance islet graft revascularisation.

1.5.3.1 Importance of islet culture for islet graft revascularisation

Experimental studies have shown that transplanted islets are revascularised by blood vessels from the host organ via the process of angiogenesis (Vajkoczy et al., 1995), but also that donor intraislet ECs contribute extensively to blood vessel formation (Vajkoczy et al., 1995, Linn et al., 2003b, Brissova et al., 2004, Nyqvist et al., 2005). The isolation-induced disruption to the vasculature leads to the loss and/or de-differentiation of intraislet ECs during the subsequent culture period (Nyqvist et al., 2005, Olsson et al., 2006). Freshly isolated islets appear to retain a large proportion of

the ECs present in native islets, with evidence to suggest that transplantation of freshly isolated islets causes superior vascular engraftment compared to that of cultured islets (Olsson and Carlsson, 2005). Moreover, islet culture is associated with a substantial loss of β -cell mass (detailed in Chapter Three). Improvement of islet culture conditions has the potential to increase β -cell survival, but also inraislet EC survival and/or the angiogenic potential of islets prior to transplantation.

Some studies have shown some success with this approach. For example, pretreatment of islets *ex vivo* with prolactin has been shown to enhance the angiogenic capacity of islets with subsequent improvements in vascular engraftment *in vivo* demonstrated (Johansson et al., 2009c). Interestingly, two separate studies have indicated that supplementation of the culture medium with typical angiogenic factors such as Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor (FGF), has little effect on the dramatic loss of ECs during culture (Nygqvist et al., 2005, Olsson et al., 2006), suggesting that it may be difficult to prevent EC loss in the absence of any three dimensional support as occurs *in vivo*. Thus, other strategies have included tissue engineering methods, such as coating of islets with ECs *in vitro* to form composite EC-islets (Johansson et al., 2005b). In a subsequent study by the same research group, the addition of MSCs to EC-islets was shown to enhance the formation of inraislet vessel-like structures *in vitro* (Johansson et al., 2008), indicating that MSCs have important angiogenic properties that could be utilised to promote islet revascularisation.

1.5.3.2 Modulation of the ‘angiogenic switch’ for improved revascularisation

An alternative approach utilized to improve islet graft revascularisation has been to modulate the ‘angiogenic switch’ by administering angiogenic factors, or inhibiting angiostatic factors in the graft recipient, thereby driving the balance towards a proangiogenic microenvironment and enhancing the proliferation, migration and maturation of ECs into functional vessels (Johansson et al., 2009b). This approach has shown some success through the manipulation of gene expression (Cheng et al., 2004, Zhang et al., 2004, Lai et al., 2005, Su et al., 2007, Olerud et al., 2008, Shimoda et al., 2010), through the mobilisation of angiogenic factors or delivery using various bioengineered scaffolds (Linn et al., 2003a, Hussey et al., 2009, Chow et al., 2010,

Cabric et al., 2010, Forster et al., 2011). Whilst many of the transgenic studies have been valuable for emphasizing the positive correlation between islet graft vascular density and function, it is unlikely that this will be translated in a clinical setting due to safety concerns. It is important to take into consideration the likelihood of systemic side-effects as well as the fact that the exact dose and timing of the pharmacological manipulation is likely to have a major influence on transplantation outcome with regards to both efficacy and safety.

1.5.3.3 Modulating the implantation site for improved revascularisation

Other strategies for enhancing islet revascularisation have focused on modulating the implantation site itself. For example, islets may be implanted into prevascularised chambers (Linn et al., 2003a, Knight et al., 2006, Hussey et al., 2009, Forster et al., 2011), which may limit the hypoxic stresses experienced by islets in the immediate post transplantation period. Strategies to implant the islets within ECM scaffolds have also shown some successful outcomes with regards to vascular engraftment. For example, transplantation of islets in a solubilised basement membrane preparation (Matrigel), which is rich in angiogenic factors (unless obtained as a growth factor reduced preparation), has been shown to promote graft revascularisation (Bharat et al., 2005). ECM scaffolds additionally offer the potential to either adhere angiogenic factors to their surface, or to act as a method for the entrapment of additional islet ‘helper cells’, which may themselves secrete angiogenic factors. Despite significant potential, these approaches may have limited success in the longer term, due to the fact that many of these scaffolds are biodegradable and are therefore likely to disintegrate after transplantation (Witkowski et al., 2009). However, if the use of scaffolds for islet transplantation can enhance vascular engraftment during the early post transplantation period, it could still be expected that this would have a positive influence on longer term transplantation outcome, due to reduced hypoxia-associated cell death. The biodegradable nature of many of these scaffolds raises the interesting possibility that attempts to co-transplant a cellular alternative with the capacity to secrete ECM proteins or enhance the ability of cells at the transplantation site to do so may provide an alternative method for positively modulating the microenvironment of the implantation site.

1.5.3.4 Co-transplantation with ‘islet helper cells’ for improved revascularisation

A number of studies have demonstrated improved islet graft revascularisation and transplantation outcome with various cell co-transplantation strategies, as detailed in Table 1.1. Candidate ‘islet helper’ cells are generally chosen because they have the potential to exert trophic effects on islet cells, thus many of these cell types have also been investigated *in vitro* for their capacity to improve islet function using a co-culture system. In a co-transplantation setting, ‘islet helper’ cells should be expected to enhance islet function and/or reduce islet cell death associated with inadequate vascular engraftment and inflammatory stresses during the post transplantation period. Cell co-transplantation strategies have the potential to increase the survival of remnant intraislet donor ECs during the post transplantation period, as well as increasing the recruitment of host ECs from the implantation site into the transplanted islet tissue, thus exerting a positive influence on the two major sources of ECs contributing to islet graft revascularisation (Brissova and Powers, 2008).

<u>Cell type</u>	<u>Therapeutic property</u>
Bone marrow cells	Enhanced VEGF expression in co-transplanted BM cells/increased islet graft revascularisation, with associated improvements in glycaemic control (Sakata et al., 2010, Bell et al., 2011)
MSCs	Release of angiogenic factors/increased islet graft revascularisation, with associated improvements in glycaemic control (Figliuzzi et al., 2009, Park et al., 2010, Sordi et al., 2010) Differentiation into ECs/increased islet graft revascularisation, with associated improvements in glycaemic control (Ito et al., 2010)
Adipose tissue-derived stem cells	Enhanced islet graft revascularisation and associated improvements in glycaemic control (Ohmura et al., 2010).
Hepatic stellate cells	Enhanced islet graft vascularisation and associated improvements in glycaemic control (Yin et al., 2007)
Vascular ECs	Enhanced graft vascularisation and associated improvements in glycaemic control (Song et al., 2010, Pan et al., 2011)

Table 1.1 Cellular candidates for co-transplantation strategies to enhance islet graft revascularisation and function.

The bone marrow is a valuable source of stem cells giving rise to haematopoietic stem cells, endothelial progenitor cells and MSCs (Pittenger et al., 1999). Co-transplantation of bone marrow cells in diabetic mice was recently shown to enhance islet revascularisation and function (Sakata et al., 2010). Notably, in this study, it was not determined exactly which subset of the bone marrow cells were responsible for this effect and the authors proposed that it was likely to be bone marrow-derived MSCs. Indeed, MSCs derived from a number of sources have been shown to enhance islet graft revascularisation. The proangiogenic effects of MSCs have largely been attributed to MSC-secreted angiogenic factors, which are likely to favour an enhanced rate or overall extent of islet revascularisation and the therapeutic properties of MSCs are described in more detail in section 1.6.2. Other tissue stem cells also offer potential for co-transplantation strategies; however, the accessibility of tissue-specific

cells is far more limited than that of bone marrow cells, making it difficult to obtain large numbers of these cell-types for clinical purposes. Co-transplantation of vascular ECs has also recently been shown to increase the microvascular EC density within transplanted islets, as well as graft function (Pan et al., 2011). It was not clear from this study whether this was due to the incorporation of the co-transplanted ECs into the newly formed vasculature of the islets, or whether the ECs were having an alternative effect. Notably, one of the difficulties of co-transplanting ECs is that they are difficult to isolate and culture in large quantities due to their low proliferation capacity, as well as their limited lifespan (Favaro et al., 2005). In addition to islet co-transplantation strategies, other attempts have been made to enhance the mobilisation of endothelial progenitor cell from the host's bone marrow, as an additional but minor source of ECs (Brissova and Powers, 2008), which can help to enhance islet graft revascularisation (Contreras et al., 2003, Miller et al., 2008).

MSCs offer a number of advantages over other cell types for islet co-transplantation strategies; in particular, the accessibility of MSCs and their capacity to be expanded extensively *in vitro* makes them a more clinically relevant cellular candidate for improving allogeneic islet transplantation. Additionally, they are 'hypoimmunogenic' meaning that they are not recognised by the host's immune system and thus do not contribute to the likelihood of graft rejection. Furthermore, their positive functional characteristics are multiple, positively influencing not only revascularisation, but also β -cell function, survival and graft rejection (see section 1.6.2), which is not a common feature of other cell types.

1.6 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are pluripotent stromal cells, with the potential to give rise to cells of diverse lineages (Pittenger et al., 1999). The pioneering work of Friedenstein and colleagues lead to the identification (Prockop, 1997) of plastic adherent cells termed colony forming unit fibroblasts, residing within the bone marrow, with the ability to differentiate into bone (Friedenstein et al., 1966). The finding that these cells were in fact able to differentiate into other mesodermal cell lineages, including tendocytes, chondrocytes and myoblasts lead to the popularisation of the term 'Mesenchymal Stem Cells' by Caplan and colleagues (Caplan, 2007) and

the ability of cells to differentiate into adipocytes, chondroblasts and osteoblasts *in vitro* now forms part of their classification as MSCs (Dominici et al., 2006). MSCs typically have a spindle-shaped fibroblast-like morphology and their classification also requires that they are plastic-adherent when maintained in standard culture conditions and that they express typical cell surface markers. MSCs should also lack the expression of typical hematopoietic and EC markers (Dominici et al., 2006). However, it is noteworthy, that the classification of human and murine MSCs does vary to some extent (Abdi et al., 2008) and the levels of expression of specific markers can vary between MSC populations, particularly after extended subculture (Meirelles et al., 2006). Table 1.2 summarizes the markers used to characterize human and murine MSCs.

	<u>Human MSCs</u>	<u>Murine MSCs</u>
<u>FACS markers</u>		
CD10	-	+/-
CD11b	-	-
CD13	+	+/-
CD29	++	++
CD31	-/+	-
CD34	-	-/+
CD44	++	++
CD45	-	-
CD73	++	0
CD90	++	+/-
CD105	++	+
CD106	++	+
CD117	-/+	-
Stro-1	+	+
Flk-1	+/-	-
Sca-1	-	+/-

Table 1.2 Markers used to characterize human and murine MSCs. CD, cluster of differentiation; FACS, fluorescence-activated cell sorting; +, positive cells; -, negative cells; 0, not determined (this table is adapted from Abdi et al., 2008).

The bone marrow contains two sources of stem cells; hematopoietic stem cells (HSCs), which renew components of the blood and MSCs which replace mesenchymal tissues. Although bone marrow MSCs represent a rare population of cells making up only 0.001-0.01 percent of all nucleated cells they can be extensively expanded in an undifferentiated state *in vitro* (Pittenger et al., 1999, Kopen et al., 1999) and grown up to cell numbers that are clinically efficacious. MSCs were initially isolated from the stromal fraction of the bone marrow (Prockop, 1997), however it has now become clear that they reside in the stroma of virtually all vascularised post natal tissues (Meirelles et al., 2006). MSCs are classified as stromal cells, which are cells of non-lymphoid origin, forming the framework of each organ; they also have the capacity to secrete stromal components (Chen et al., 2007). This subset of adult stem cells plays an important role in tissue repair and regeneration at

these sites. By expressing various molecules, MSCs can support the adhesion, proliferation and survival of distinct cell subsets (Uccelli et al., 2008). Most adult tissues additionally contain reservoirs of tissue specific stem cells that can contribute to tissue repair and maintenance following some form of trauma, ageing or disease process.

1.6.1 Source of Mesenchymal Stem Cells

MSCs can be derived from virtually all vascularised post natal tissues including the brain, spleen, liver kidney, lung, bone marrow, muscle, thymus and pancreas (Meirelles et al., 2006). It is thought that the distribution of MSCs is related to their existence in a perivascular niche, with evidence suggesting that they are resident in vascular walls (Meirelles et al., 2006), thus supporting growing evidence for their relationship with pericytes (Farrington-Rock et al., 2004). Under normal physiological conditions MSCs appear to play a role as vascular supportive cells. In general, MSCs derived from different tissues have a very similar morphology and immunophenotype to bone marrow derived MSCs. However, there is some evidence of variable differentiation capacities, suggesting tissue specific functions for different MSC populations (Meirelles et al., 2006, Keyser et al., 2007). It is also important to consider that the functional properties and differentiation potential of stem cells is influenced by extracellular cues and the local microenvironment, as well as the intrinsic genetic programs within the cells (Li and Xie, 2005). MSCs derived from different tissue sources have been shown to secrete varying levels of trophic factors and have different functional properties (Park et al., 2009, Veriter et al., 2011), thus at present we cannot predict the efficacy of MSCs derived from different sources to improve islet transplantation outcome with any certainty. Although, MSCs derived from the kidney, pancreas and bone marrow have all been shown to have a positive influence on transplantation outcome in diabetic rodents or non-human primates (see Table 1.3), indicating a certain degree of flexibility in the choice of tissue source.

Kidney-derived MSCs were used in all experiments presented in this thesis. The MSCs were kindly provided by Pedro Chagastelles (Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil), who was able to derive mouse MSCs from the bone marrow, pancreas, liver and kidney. The kidney MSCs were of particular

interest because at the time of planning the experiments, there were already reports that bone marrow derived MSCs could improve engraftment and graft function in a syngeneic mouse model of diabetes (Figliuzzi et al., 2009), so we were interested in looking at alternative sources. We chose the kidney MSCs above liver or pancreas because they had the best growth kinetics, but also because we were planning to implant the islets + MSCs beneath the renal capsule and therefore hypothesised that the MSCs may exert more of a positive influence if they were localised to the tissue from which they were derived.

As well as the tissue source, it is also important to consider whether the MSCs are likely to be most efficacious if host, donor or third-party derived. Abdi and co-authors recently reported comparable immunosuppressive properties of MSCs from different donors, including ‘third party’ donors (Abdi et al., 2008). Given that the microenvironmental niche is important for the functional properties of MSCs, it could be expected that disease states would affect these properties. Phadnis and colleagues recently demonstrated that bone marrow MSCs from diabetic patients had similar biological characteristics in terms of morphology, phenotype and multilineage potential, to those of non-diabetic control (Phadnis et al., 2009), indicating that patient-derived MSCs may have therapeutic potential. However, other studies have indicated that disease states, such as chronic renal failure (Zhao et al., 2007) and rheumatoid arthritis (Garayoa et al., 2009) can adversely affect the biological properties of MSCs. Notably, the frequency of MSCs in the bone marrow has also been shown to decline with age (Fibbe, 2002). Thus donor variability is an important consideration when predicting the potential for MSCs to improve transplantation outcome. Future directions for MSC research should certainly aim to standardise the assessment of MSC preparations, with particular reference to the importance of passage number, culture conditions and source of MSC (Abdi et al., 2008). Despite this, the capacity for MSCs to replicate extensively *in vitro* as undifferentiated cell types, along with their multilineage potential (Pittenger et al., 1999), makes them excellent candidates for regenerative medicine and in particular cell replacement therapies.

1.6.2 Mesenchymal Stem Cells and β -cell replacement strategies

The capacity for MSCs to differentiate into cells of different lineages means that they have emerged as a useful tool for a number of clinical applications involving tissue engineering or cell replacement (Bianco and Robey, 2001, Jiang et al., 2002). MSCs have been investigated in a variety of disease models including T1DM for a number of reasons, which include: 1) their capacity for tissue regeneration (Sordi and Piemonti, 2010); 2) their anti-inflammatory and immunomodulatory properties (Uccelli et al., 2008, Abdi et al., 2008, Caplan, 2009) and; 3) the positive paracrine influences they are capable of exerting upon adjacent cells including islet cells through the secretion of bioactive molecules (Xu et al., 2008). Emerging evidence suggests that MSCs derived from different tissue sources can help to improve the outcome of islet grafts in diabetic recipients, with most therapeutic mechanisms involving beneficial effects on engraftment and the immune system, as shown in Table 1.3.

<u>Tissue origin</u>	<u>Therapeutic mechanism</u>
Bone marrow	Enhanced engraftment and graft function (Figliuzzi et al., 2009, Ito et al., 2010). Enhanced engraftment and function, reversal of rejection (Berman et al., 2010). Improved islet graft protection and function through immunosuppressive effects/delayed rejection (Jacobson et al., 2008, Solari et al., 2009, Ding et al., 2010, Longoni et al., 2010, Li et al., 2010).
Kidney	Immunomodulatory effects/delayed graft rejection (Huang et al., 2010).
Pancreas	Enhanced engraftment and graft function (Sordi et al., 2009).

Table 1.3 Therapeutic mechanisms through which MSCs derived from different tissues can improve the outcome of islet grafts for the treatment of diabetic hyperglycaemia.

Recent experimental studies have emphasized the positive influence that MSCs can exert on islet function and survival in syngeneic islet transplantations, thus making better use of the donor islets that are available. Additionally, studies have focused on the positive effects MSCs exert in an allogeneic transplantation setting, with evidence

to suggest that MSC infusions or co-transplantation with islets can delay or prevent graft rejection, as discussed in section 1.6.3 below. Thus, research to date highlights the capacity for MSCs to help in addressing the major obstacles, which limit the widespread application of islet transplantation: 1) the scarcity of islets available for transplantation; 2) the high rates of islet graft failure; and 3) the need for life-long immunosuppression.

1.6.3 Immunomodulatory properties of Mesenchymal Stem Cells

Treatment of T1DM should ideally address both the insulin deficit and also the autoimmune response to the cells expressing insulin (Aguayo-Mazzucato and Bonner-Weir, 2010). Therefore, the immunomodulatory properties of MSCs are of great interest in the context of islet transplantation (Uccelli et al., 2008, Jacobson et al., 2008, Ding et al., 2009, Solari et al., 2009, Boumaza et al., 2009, Longoni et al., 2010). MSCs have been shown to create a beneficial microenvironment through the secretion of anti-inflammatory and immunosuppressive molecules, which can modulate the host's immune system in a positive way, thus helping to prevent allogeneic graft rejection. Additionally, the immunosuppressive properties of MSCs are exerted through direct cell-cell interactions with T- and B-cells, as well as natural killer and dendritic cells (Ryan et al., 2005b, Nauta et al., 2006). MSCs are also able to increase the numbers of CD4⁺CD25⁺ and CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Selmani et al., 2008) and reduce the proliferation of both CD4⁺ and CD8⁺ T cells (Le Blanc et al., 2003), which could potentially reduce or prevent recurrent autoimmunity. MSCs have also been described as hypoimmunogenic cells, due to their lack of expression of MHC class II molecules and most of the classical co-stimulatory molecules (Tse et al., 2003). This is beneficial in terms of immune rejection as it enables them to escape recognition by the host's immune system. In theory, MSCs could reduce or prevent the need for life-long immunosuppression, which has deleterious effects on transplanted islet cells (Andersson et al., 1984, Rosenberg et al., 1991, Redmon et al., 1996), whilst producing unwanted side effects for the patient. In support of this hypothesis, Longoni et al have demonstrated the synergistic effect of MSCs with immunosuppressive drugs in mice (Longoni et al., 2010) and there is also evidence to suggest that intravenous MSC infusions may help to prevent allogeneic graft rejection in non-human primates (Berman et al., 2010).

MSCs are not mutually exclusive in their ability to show potent immunomodulatory potential. Fibroblasts have emerged as a cellular candidate with immunosuppressive properties, including the ability to suppress T-cell proliferation *in vitro* (Haniffa et al., 2009). Hepatic stellate cells have also shown the capacity to suppress the rejection of allogeneic islet grafts (Yin et al., 2007), however, the accessibility of MSCs and their capacity to be expanded extensively *in vitro*, as previously mentioned, means that they may be a better cellular source for improving allogeneic islet transplantation with regards to the prevention of graft rejection. Prochymal, a formulation of immunomodulatory MSCs is also currently being used for disease management in clinical trials, as discussed in section 1.6.6.

1.6.4 Regenerative properties of Mesenchymal Stem Cells

The regenerative properties of MSCs, as well their capacity to promote tissue repair, mean that they have the potential to aid the engraftment of transplanted islet cells, which are subjected to a number of stresses during the isolation, pre-transplant and post-transplant period (Linn et al., 2006). Additionally, the capacity of MSCs to migrate specifically to injured tissues (Barbash et al., 2003, Lee et al., 2006, Fox et al., 2007), including islets (Sordi et al., 2005), means that they have the potential to increase the regeneration of any remaining endogenous β -cells in the pancreas.

Adequate revascularisation is essential for the regeneration of any vascularised tissue, thus the angiogenic properties of MSCs alone are likely to have a positive influence on the engraftment of transplanted islets. The release of anti-inflammatory cytokines, anti-apoptotic, angiogenic and mitogenic factors all function together to create a niche, which is permissive for the repair of damaged tissue. Additionally, MSCs show tremendous phenotypic plasticity, with the ability to acquire tissue specific characteristics when subjected to the appropriate stimuli (Choi et al., 2005, Lange et al., 2005). Thus, recent reports have suggested that MSCs have the capacity to transdifferentiate into ECs at the graft site (Ito et al., 2010), which may potentially enhance islet graft revascularisation by providing an additional source of ECs. Moreover, studies have suggested that MSCs may be able to transdifferentiate into β -cells or insulin producing cells when provided with the appropriate stimuli (Ianus et

al., 2003, Tang et al., 2004, Chen et al., 2004, Sun et al., 2007, Xie et al., 2009, Yuan et al., 2010), although these reports are not supported by others (Sordi et al., 2010).

Studies have shown that MSCs express chemokine receptors that enable them to migrate towards islet-secreted chemokines (Sordi et al., 2005), thus partially explaining reports that MSCs have the ability to migrate to injured tissues (Ezquer et al., 2008). Interestingly, it has been suggested that hypoxia can also increase the migratory capacity of MSCs (Hung et al., 2007b), which may be important during the immediate post transplantation period when the islets are most vulnerable to hypoxic stresses (Davalli et al., 1996, Biarnes et al., 2002, Miao et al., 2006). The migratory capacity of MSCs is important as it may allow some flexibility in the route of MSC administration. It also implies that repeated intravenous infusions of MSCs may have possible therapeutic efficacy, as supported by the Berman study where repeated intravenous MSC infusions after islet-MSC co-transplantation were shown to prevent allogeneic graft rejection (Berman et al., 2010). At present, there is conflicting evidence regarding the capacity of MSCs to cause endogenous pancreatic β -cell regeneration, with some studies supporting this as a therapeutic mechanism of MSCs (Lee et al., 2006, Dong et al., 2008, Ezquer et al., 2008) and others that do not (Urban et al., 2008, Berman et al., 2010). There is also some evidence to suggest that under strict conditions of normoglycaemia following induction of diabetes by high dose STZ-administration into rats (60mg/kg of body wt); the few surviving β -cells that remain in the pancreas can regenerate, in the absence of MSCs or any other intervention (Jorns et al., 2001). Additionally, emerging evidence suggests that α -cells remaining in pancreas are able to spontaneously acquire β -cell characteristics following near total β -cell destruction (Thorel et al., 2010). Thus, emphasizing the importance of nephrectomising cured mice in both control islet-alone and islet + MSC transplant groups, to ensure that any conclusions regarding graft function are accurate and do not reflect any significant differences in the number of endogenous β -cells within the pancreas of either transplant group.

The regenerative properties of MSCs make them a particularly attractive candidate for improving the efficacy of clinical islet transplantation in recent onset diabetic patients (disease duration ≤ 18 months), where there are more residual β -cells remaining and

there is also an increased rate of β -cell proliferation, in contrast to longer term diabetics (6-12yr), where β -cells no longer proliferate (Willcox et al., 2010), if indeed there are any residual β -cells left at all. A final, but important consideration regarding the regenerative properties of MSCs is that they may have therapeutic potential for the treatment of diabetic complications as reviewed recently by Volarevic and colleagues (Volarevic et al., 2011).

1.6.5 Paracrine properties of Mesenchymal Stem Cells

Paracrine interactions between cells occur when a secretory product moves a short distance through the interstitial fluid to reach a target cell (Weir and Bonner-Weir, 1990). MSCs have been shown to secrete a number of soluble cytoprotective, angiogenic, mitogenic, anti-inflammatory and immunomodulatory molecules, which mediate trophic effects on the target cell (Xu et al., 2008). Notably, the paracrine influences of MSCs are likely to play an important role in their ability to promote tissue regeneration and repair, as discussed above (see section 1.6.4), but are distinct from the capacity for MSCs to differentiate into injured tissues (Petersen et al., 1999, Woodbury et al., 2000). The secreted bioactive factors may have a direct effect, influencing the target cell themselves (i.e. the β -cell), or indirect by modulating the activity of surrounding cells (such as ECs).

A number of studies have demonstrated the importance of soluble mediators, by utilizing a transwell co-culture system in which direct cell-cell contact between islets and MSCs can be prevented, but the diffusion of paracrine factors is allowed due to the separation of these cell types using a semi-permeable membrane (Di Nicola et al., 2002, Tse et al., 2003, Rasmusson et al., 2003, Park et al., 2009, Jung et al., 2011). Additionally, MSC-conditioned media experiments can also help in determining the importance of MSC-secreted factors (Park et al., 2010, Jung et al., 2011).

In a co-transplantation setting, soluble bioactive mediators secreted by MSCs may potentially improve vascular engraftment, enhance islet function and survival, as well as prevent immunological rejection, as represented in Figure 1.4.

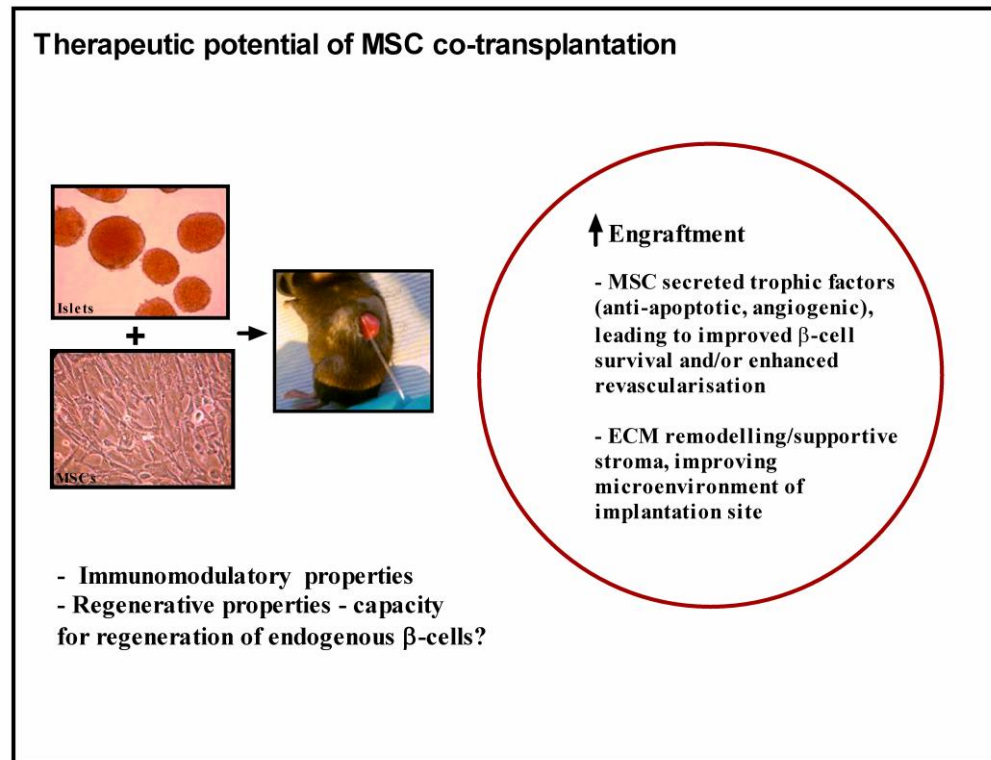


Figure 1.4 Schematic of the therapeutic potential of MSC co-transplantation.

Several groups have demonstrated the ability of MSCs derived from different tissues to secrete angiogenic factors, such as VEGF, HGF and IL-6 both *in vitro* (Park et al., 2010, Sordi et al., 2010) and *in vivo* (Ito et al., 2010), which correlated with enhanced vascular engraftment *in vivo*. Additionally, MSC-secreted matrix metalloproteases (MMPs) enhance the functional capacity of ECs to form blood vessels (Johansson et al., 2008). This is likely to be because MMPs can help to degrade ECM proteins (Ghajar et al., 2006, Potapova et al., 2007), thus paving the way for the migration of ECs. Similarly, experimental data supports the hypothesis that MSCs may support islet graft function and survival by secreting immunomodulatory cytokines, which modulate the immune system to prevent graft rejection (Ding et al., 2009, Solari et al., 2009, Longoni et al., 2010, Berman et al., 2010) and inflammation mediated-islet cell dysfunction and death. Amongst the many soluble mediators indicated, TGF- β , HGF, indoleamine 2,3-dioxygenase (IDO), MMP-2 and MMP-9 seem to be important and have been shown to suppress T and B cell activation and/or proliferation (Di Nicola et al., 2002, Meisel et al., 2004, Krampera et al., 2006, Ding et al., 2009).

The isolation procedure disrupts the complex cytoarchitecture of islets and causes the loss of trophic support from the surrounding ductal cells (Ilieva et al., 1999), ECM proteins (Wang and Rosenberg, 1999) and that provided by ECs lining the capillaries of the islet vasculature (Johansson et al., 2009a). Therefore, the additional trophic factors provided by MSCs are particularly important during the immediate post transplantation period before the revascularisation process is completed, as well as during the pretransplant period where the islets are subjected to hypoxic stresses during culture (Lau et al., 2009). Thus, the paracrine mechanisms, through which MSCs have been shown to improve islet transplantation outcome, should also be considered as a potential way to improve islet quality, function and survival during the pretransplant period. Recent studies have indicated that MSC-derived trophic factors can improve islet quality after culture, as shown through reduced ADP/ATP ratios and DNA fragmentation, enhanced glucose-stimulate insulin secretion and increased expression of the anti-apoptotic molecules Bcl-xL and Bcl-2 (Park et al., 2009) *in vitro*. Additionally, STZ-induced diabetic mice transplanted with islets precultured in MSC-conditioned media had better glycaemia than control mice (Park et al., 2010), which should be expected as the quality of islets prior to transplantation is thought to have an important impact on the success of islet transplantations (Boker et al., 2001, Robertson, 2004).

The paracrine activities of MSCs are known to be influenced by the microenvironment in which they reside. Recent studies suggest that pro-inflammatory cytokines, such as IFN- γ , IL1- β and TNF- α , which are present during the immediate post transplantation period (Alejandro et al., 1986, Nagata et al., 1990, Barshes et al., 2005, Montolio et al., 2007), can upregulate the immunosuppressive properties of MSCs (Ryan et al., 2007, Krampera et al., 2006). Hypoxia is another important stimulus, which is thought to stimulate the angiogenic potential of MSCs (Annabi et al., 2003, Hung et al., 2007a, Efimenko et al., 2011). Thus, the paracrine activities of MSCs are likely to be highly dependent upon the specific conditions in which the MSCs are cultured or those where the MSCs localise to *in vivo*.

It is clear that there are a number of paracrine mechanisms by which MSCs exert their therapeutic efficacy in animal models of diabetes, however, it has additionally been

suggested that direct cell-cell contact is important for maximising the beneficial effects of MSCs on islet function (Jung et al., 2011). Efforts to increase MSC-islet interactions through direct-contact culture methods or co-transplantation strategies may therefore make the best use of available islets and MSCs.

1.6.6 Clinical trials and safety issues concerning the use of Mesenchymal Stem Cells

Numerous preclinical and clinical studies have demonstrated the safety and toxicity free-effects of MSC transplantation or intravenous infusion in different diseases, including cardiovascular disease, renal disease, Crohn's diseases, cirrhosis, osteogenesis imperfect, graft-versus-host disease and T1DM, as detailed at <http://www.ClinicalTrials.gov>. A partnership between industry experts Osiris and the Juvenile Diabetes Research Foundation (JDRF) has recently been formed and is investigating the potential for MSCs to help in the treatment of recent onset T1DM. Intravenous infusions of Prochymal (alone); a formulation of adult BM MSCs were given in this phase II clinical trial to test the safety and efficacy, but also to determine whether there is any effect of MSCs on the c-peptide AUC response, total daily insulin dose, HbA1c, number of severe hypoglycaemic episodes and changes in the level of IA-2 and GAD auto antibodies. Thus, the outcomes of this trial appear to be investigating whether Prochymal can enhance the survival and/or increase the regeneration of residual endogenous pancreatic β -cells, which may be at least in part through reducing/preventing recurrent autoimmunity. Additional clinical trials are currently recruiting for islet-MSC co-transplantation studies.

It is important to consider the potential safety issues that may arise from the clinical use of MSCs, which by their very own definition are known to show phenotypic plasticity and hence could have the potential to form tumours in patients. A number of studies with murine MSCs have shown that they can undergo malignant transformation following culture (Miura et al., 2006, Aguilar et al., 2007), but the risks associated with culturing human MSCs seem to be lower (Miura et al., 2006, Aguilar et al., 2007, Tolar et al., 2007). Berman and colleagues showed that there were no chromosomal abnormalities in MSCs derived from the iliac crest aspirate of nonhuman primates through to passage 11 (Berman et al., 2010), supporting

observations for human MSCs (Bernardo et al., 2007). To date there have been no reports of any malignant transformation of human MSCs in clinical trials aiming to treat autoimmune diseases (Abdi et al., 2008). However, the extent to which the capacity for tumour formation varies between species, tissue source and passage number is currently unknown, emphasizing the need for increased quality control testing and standardised characterisation of MSC phenotype and functional properties. Furthermore, there is currently little information regarding the longer term effects of MSCs, with regards to tumourogenicity, but also the potential concern that MSCs may transdifferentiate into unwanted tissues, such as bone. For example, a recent study by Duprez and colleagues showed that syngeneic transplantation of their mouse MSC-islets resulted in bone formation underneath the kidney capsule (Duprez et al., 2011). However, it was noted that human MSCs are less likely to differentiate into bone *in vivo* unless the appropriate stimuli are present.

Despite some of the safety concerns regarding the use of MSCs clinically, it is clear that MSCs have a number of functional properties that make them excellent candidates for improving islet transplantation outcome: 1) They can be derived from virtually all vascularised post natal tissue, which may increase the supply of these cells for clinical use; 2) They can be rapidly expanded *in vitro* to numbers that are clinically relevant; 3) They are ‘hypoimmunogenic’ avoiding recognition by the host immune system; and 4) In addition to their positive cytoprotective and angiogenic effects, they also have the potential to help prevent graft rejection and recurrent autoimmunity. Although other cell types have been shown to have positive trophic or immunological effects on islet graft function, the capacity for these cell types to be expanded *in vitro* to clinically efficacious numbers is more limited. Furthermore, most other cell types do not inherently possess all of these major functional benefits, which together have great potential to address a large number of the problems associated with clinical allogeneic islet transplantation. Thus, MSCs have good potential to maximise the use of the donor islets that are available, which taken together with their immunomodulatory functions, could potentially enable the more widespread application of allogeneic islet transplantation as a treatment for T1DM.

1.7 Aims and experimental objectives

Disruption to the complex cytoarchitecture of the islets as a result of the isolation process and the culture period that follows causes a loss of valuable islet material for transplantation. Furthermore, the microenvironment to which islets are transplanted experimentally and clinically does not provide them with the necessary trophic support, which causes further islet dysfunction and loss. The loss of ECs during culture and inadequate revascularisation contribute to this β -cell loss and inadequate function. Therefore, a series of experimental studies were carried out to initially characterise the extent to which the islet vasculature is disrupted after culture and transplantation. Subsequent experiments utilised MSCs to determine whether their therapeutic properties could improve transplantation outcome:

- A syngeneic mouse model of diabetes was used to determine the effect of co-transplanting MSCs on: 1) the morphological and vascular engraftment of islets at one month post transplantation; and 2) graft function over a one month monitoring period.
- The same syngeneic mouse model of diabetes was used to determine whether co-transplantation of MSCs had the potential to increase the rate, as well as the overall extent of islet revascularisation.
- Islets were precultured with MSCs to assess whether MSCs have any effect on EC and/or β -cell survival and function *in vitro* and whether this correlates with graft function *in vivo*.

Additional experiments tested the hypothesis that maintaining normal islet size and morphology after transplantation in the absence of MSCs or any other cell type would enhance islet revascularisation and/or graft function:

- Islets were spread beneath the renal capsule and islet function and vascular engraftment were assessed, in comparison to the control group in which islets were transplanted in the conventional way as a single islet pellet.

- Islets were implanted beneath the renal capsule in matrigel plugs to prevent the fusion of individual islets, as typically occurs at this implantation site and graft function assessed.

2 Chapter Two - Materials and Methods

2.1 Isolation of mouse islets of Langerhans

Islets were isolated from male ICR or C57BL/6 mice killed by cervical dislocation. 2-3ml of cold collagenase solution (from *Clostridium histolyticum*, type X1, Sigma-Aldrich, Poole, UK; 1mg/ml Modified Eagle's Medium (MEM), Sigma-Aldrich) was injected into the pancreas via the common bile duct following clamping at the ampulla of Vater. The distended pancreas was then removed and incubated in a stationary water bath at 37°C for 10 min. After this enzymatic digestion, the pancreatic islets were washed in MEM supplemented with 10% Newborn Calf Serum (NCS) and 100U/ml penicillin + 0.1mg/ml streptomycin to stop the digestion and remove any remaining collagenase solution. After the second wash (340 x g, 10°C, acceleration speed 9, brake speed 9, for 1.5 min), the pancreatic tissue was vortexed and passed through a sieve in order to discard as much of the exocrine and other contaminating tissue as possible. In order to obtain a pure preparation of islets, a purification gradient was set up by adding Histopaque® (a polysucrose solution with a density of 1.077g/ml) to the pancreatic tissue. 15ml of Histopaque® for 2-3x pancreata in a 50ml falcon tube was used. The pancreatic tissue in histopaque was vortexed briefly and 10ml of MEM + supplements was then added slowly using a 10ml pipette. The islets were separated from the exocrine tissue by a centrifugation density gradient at 1900 x g, 10°C for 24 min, with slow acceleration (speed 1) and no brake (speed 0). Islets were removed from the interphase of the histopaque and MEM and washed three times. Islets were then handpicked into 60mm petri dishes (unless stated otherwise) containing 5ml of RPMI supplemented with 10% Foetal Bovine Serum (FBS) and 100U/ml penicillin + 0.1mg/ml streptomycin.

2.2 Culture of mouse islets of Langerhans

Islets isolated from ICR or C57BL/6 mice were cultured as groups of approximately 150 islets in 60mm sterile bacterial petri dishes (unless stated otherwise), using RPMI supplemented with 10% FBS, 100U/ml penicillin + 0.1mg/ml streptomycin, with a

glucose concentration of 11.1mmol/l and maintained in a humidified incubator (37°C, 95% air/5% CO₂). The media was changed every second day.

2.3 Animals and surgical procedures

Inbred male C567BL/6 mice (Harlan, Huntingdon, UK) weighing 20-30g were used for all syngeneic islet transplantations. Male ICR mice (Harlan) weighing 25-30g were used for the majority of *in vitro* studies, unless stated otherwise. All procedures were performed under the Home Office approved project licence (PPL no. 70/6770) and personal licence (PIL no. 24696). All animal procedures were carried out according to our institution's ethics committee. Animals had free access to water and pelleted food throughout experiments (except for when fasting prior to glucose tolerance tests).

2.3.1 Induction of diabetes by streptozotocin injection

Mice were made diabetic with a single injection of streptozotocin (STZ) (Sigma-Aldrich). STZ and citrate buffer aliquots were kept on ice until immediately before needed and freshly dissolved STZ in citrate buffer was administered intraperitoneally (i.p.) at a concentration of 180/mg/kg body weight, within 10 min. Only mice with a non-fasting blood glucose concentration of ≥ 20 mmol/l 3-4 days later were used as diabetic recipients.

2.3.2 Minimal islet mass model

STZ-diabetic C56Bl/6 mice were used as graft recipients. The number of islets transplanted aimed to cured diabetes in only a proportion of diabetic recipients (approximately fifty percent). Animals were considered cured if their non-fasting blood glucose concentrations were lower than 11.1mmol/l on at least two consecutive days, without reverting to diabetes at any subsequent time point. A treatment was considered as having a positive influence on graft function/transplantation outcome if it increased the proportion of recipients who cured and/or the time-to-cure (rate of diabetes reversal) was improved.

2.3.3 Islet Transplantation

All surgical tools were cleaned thoroughly and autoclaved before use. Islets were counted into groups of 150-200 (as specified in individual results chapters) and washed in sterile RPMI-1640 (Sigma-Aldrich). Islets were kept on ice before centrifuging in PE50 polyethylene tubing (Becton Dickinson, Sparks, MD) to form pellets (unless stated otherwise). In order to do this, approximately 500µl of sterile RPMI was taken up into the Hamilton syringe to ensure that there was no dead space. Islet aliquots were then aspirated into a 200µl pipette tip, which had been cut at the top to form a connection with the Hamilton syringe (Fisher, Pittsburg, PA) to which it was attached. The tip was then connected with the PE50 polyethylene tubing and the islets injected ~5cm into the tubing. The tubing was then folded just below the islets and held in place using connector tubing. The pipette tip with attached tubing was then removed from the Hamilton syringe for centrifugation. The fold in the tube prevented islets from passing this point during the subsequent centrifugation step (2 min at 200 x g), used to pellet the islets. The pipette tip was then topped up with medium to prevent any air bubbles forming and re-attached to the Hamilton syringe. The pellet could then be seen at the point at which the fold was present in the tubing and the connector tubing here was removed. The Hamilton syringe was then used to draw the islet pellet back ~1cm from the fold and the tubing was then cut immediately in front of the islet pellet.

Male C57BL/6 mice were anaesthetised using isofluorane and buprenorphine administered at a dose of 0.03 mg/kg, as an analgesic. A lumbar incision was made and the left kidney was then exposed, externalised and an incision in the capsule made by dragging a 23G needle across the surface, so that the islets could be implanted underneath the kidney capsule, as shown in below in Figure 2.1. Sterile saline was used to prevent the kidney from dehydrating. A Hamilton syringe and PE50 polyethylene tubing was used to implant the islet graft. The kidney was then placed back into the peritoneal cavity and the lumbar incision and overlying skin was then sutured using a needle holder and 4-0 VICRYL (Ethicon, Johnson and Johnson). Mice were monitored during recovery from the surgical procedure and kept in a 37°C incubator during this time.

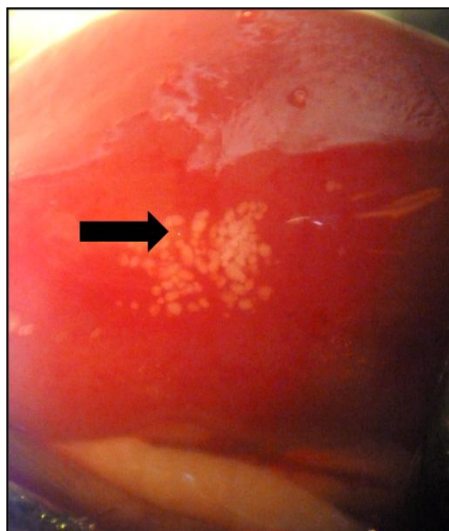


Figure 2.1 Photograph of islet graft-bearing kidney. Islet pellets were transplanted beneath the kidney capsule. Arrow indicates the islet pellet immediately after transplantation.

2.3.4 Animal monitoring

Non-fasted blood glucose concentrations were determined using a blood glucose meter and strips (Accu-Chek; Roche, Burgess Hill, UK) with blood obtained from a pin prick to the tail (using a 27G needle). Blood glucose concentrations were taken at the same time for each reading (between 8.30 and 9.30a.m) throughout the monitoring period. Blood glucose concentrations and body weight were monitored every 2-4 days unless stated otherwise. Insulin (Caninsulin, Dunlops, Dumfries, Scotland) was administered subcutaneously on alternate days (two insulin units, using terumo[®] MYJECTOR[®] 29G x1/2'') to mice with non-fasting blood glucose concentrations ≥ 20 mmol/l to prevent excessive weight loss.

2.3.5 Glucose Tolerance Tests

Intraperitoneal glucose tolerance tests (IPGTTs) were performed at one month. All cured mice were fasted overnight for a maximum of 16 hr. Basal blood glucose was measured using an AccuChek blood glucose meter and strips (Roche, UK) prior to i.p. injection of a 30% glucose solution (see Table 2.1) administered at 2g/kg. Blood glucose measurements were then taken 15, 30, 60 and 90 and 120 minutes later.

30% glucose solution

<u>Reagent</u>	<u>Volume (ml)</u>
45% glucose solution (Sigma)	10
1x phosphate buffered saline (PBS) (Sigma)	5

Table 2.1 The 30% glucose solution was made up and sterile-filtered through 0.22 μ m filters (Millipore) prior to i.p. injection into mice.

2.3.6 Nephrectomies

The graft bearing kidneys of cured mice were removed by nephrectomy to determine whether they would subsequently become hyperglycaemic (blood glucose >20mmol/l). This is an important procedure to establish whether cured mice have done so due to the curative capacity of the implanted islet graft, or whether there has been significant endogenous β -cell regeneration in the pancreas, which in itself would be sufficient to maintain normoglycaemia.

Cured mice were anaesthetised using isofluorane and buprenorphine administered at a dose of 0.03 mg/kg, as an analgesic. A lumbar incision was made and the graft-bearing kidney was then exposed. Sterile saline was used to prevent the kidney from dehydrating. Any fibrous tissue forming attachments between the renal capsule and the wall of the posterior of abdominal cavity was carefully excised using sharp dissecting scissors. The renal artery, renal vein and ureter were clamped. A 4-0 VICRYL (Ethicon, Johnson and Johnson) was the used to tie off the renal artery vein and ureter. A disposable sterile scalpel with surgical steel blades size 10 (Swann morton limited, Sheffield, UK) was used to remove the graft bearing kidney. The clamp was then carefully removed, checking that there was not any bleeding. The lumbar incision and overlying skin was then sutured. Mice were monitored during recovery from the surgical procedure and kept in a warm incubator during this time.

2.3.7 Plasma samples for quantification of insulin

Heparin sodium (5,000 I.U./ml) was taken up into a glass pipette with plastic bulb and expelled fully, ensuring no residual liquid remained by blotting on a paper towel before collecting blood samples, in order to prevent the blood from clotting. Blood was then obtained by a pin prick (27G needle) to the tail, using the capillary action of

the pipette. Samples were kept on ice for no longer than one hour, before plasma was separated from whole blood by centrifugation at 13,000 rpm for 10 min, 4°C. 10µl of the supernatant was then used to measure plasma insulin using a mouse insulin ELISA (Mercodia, Uppsala, Sweden), according to manufacturer's instructions, as described below (section 2.4).

2.4 Plasma insulin ELISA

The Mercodia mouse insulin ELISA (Mercodia, Uppsala, Sweden) assay is a very sensitive assay with a detection limit of 0.025 µg/l. This means that the insulin concentration in plasma can be determined accurately using volumes as low as 10µl.

2.4.1 Principle of the assay

The insulin ELISA is a two-site enzyme immunoassay in which two monoclonal antibodies are directed against two separate antigenic determinants on the insulin molecule. During the incubation steps insulin in the sample binds to peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to the bottom of the microplate well. The washing step removes unbound enzyme labelled antibody and the bound conjugate is detected by reaction with 3,3',5,5'- tetramethylbenzidine. The reaction is then stopped by adding acid to give a colorimetric endpoint, which can be measured spectrometrically.

2.4.2 Method

Plasma insulin was measured using 10µl of each sample and standards/calibrators provided in the kit. All reagents were allowed to equilibrate to room temperature before starting the assay. Briefly, 10µl of each sample, calibrator and control was added to microplate wells. 100µl of enzyme conjugate 1x solution was added to each well and the whole plate incubated for 2 hr at room temperature gently shaking. The plate was washed manually with wash buffer 1x solution (provided in the kit) using a wash bottle. It was important to wash the plate adequately to ensure that all unbound enzyme labelled antibody was removed (but taking care to avoid prolonged soaking). 200µl of substrate 3,3',5,5'-Tetramethylbenzidine (TMB) was then added to each well using a multichannel pipette and the plate incubated at room temperature for 15 min.

50µl of stop solution (provided in the kit) was then added to each well and placed on a plate shaker for approximately five sec to ensure adequate mixing. The concentration of insulin in each sample was calculated from a calibrator curve generated using Ascent software for multiskan ascent version 2.4, as shown in Figure 2.2.

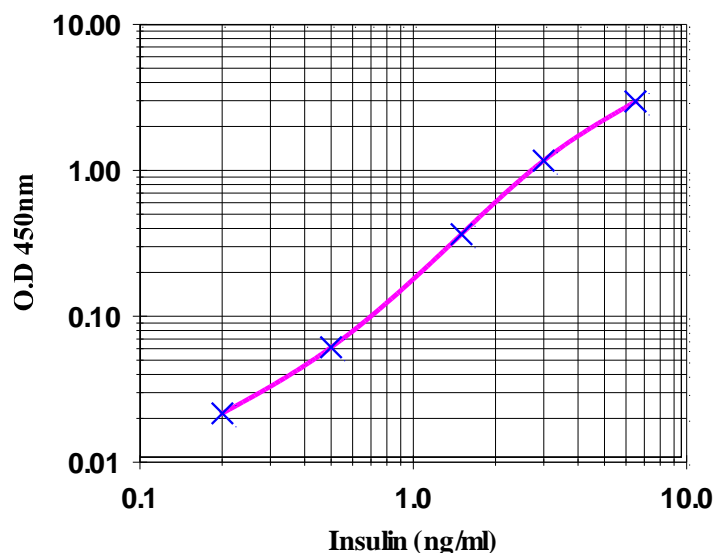


Figure 2.2 Calibrator curve used for the determination of plasma insulin concentrations. The optical density was measured at 450nm and concentration of insulin calculated from the calibrator curve, using Cubic Spline regression analysis (Ascent software for multiskan ascent version 2.4).

2.5 Mesenchymal stem cell isolation and characterisation

I am grateful to Pedro Chagestelles (Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil) for isolating and characterising the mesenchymal stem cells (MSCs) used in the studies presented in this thesis. Below is a brief description of the methods used.

2.5.1 Isolation of kidney-derived mesenchymal stem cells

Kidney-derived MSCs were isolated from C57Bl/6 mice. Kidneys were rinsed in Ca^{2+} - and Mg^{2+} - free Hanks' balanced salt solution containing 10 mmol/l sodium HEPES (Sigma-Aldrich) and cut into small pieces. The fragments were digested with collagenase type I (1 mg/ml; Sigma- Aldrich) for 30 to 45 min at 37°C and then triturated with a glass Pasteur pipette. Cells were pelleted by centrifugation for 10 min

at 400 x g at room temperature. After this, cells were resuspended in DMEM supplemented with 100U/ml penicillin + 0.1mg/ml streptomycin and 10% (vol./vol.) FCS, seeded in six-well dishes (3ml/well) and incubated at 37°C in a humidified atmosphere containing 5% CO₂. The medium was changed after 24 h, with removal of non-adherent cells. When cultures reached confluence, cells were trypsinised and subcultured in new flasks, at passage ratios empirically determined for two subcultures a week.

2.5.2 Adipogenic and osteogenic differentiation

Adipogenic differentiation was induced by cultivation of confluent cultures in DMEM containing 20% (vol./vol.) FCS, 2.5µg/ml insulin, 100µmol/l indomethacin, 5µmol/l rosiglitazone and 10nmol/l dexamethazone. For osteogenic differentiation, confluent cultures were cultivated in DMEM containing 10% FCS (vol./vol.), 10mmol/l B-glycerophosphate, 5µg/ml ascorbic acid and 10nmol/l dexamethazone. Cultures were maintained in differentiation media for one month with medium changes twice a week. Cell differentiation was analyzed by staining with Oil Red O or Alizarin Red S for adipogenic and osteogenic differentiation, respectively.

2.5.3 Immunophenotyping by flow cytometry

Kidney-derived MSCs were analysed for the presence of surface markers by flow cytometry. MSCs at the 5th passage were trypsinized, resuspended in PBS and incubated with the following FITC- or PE-conjugated antibodies: CD11b, CD31, CD44, CD45 and Sca-1 (BD Pharmingen, San Diego, CA). After 30 min incubation at 4°C the cells were washed and resuspended in 0.5ml PBS. Cells were analysed in a FACS calibur cytometer equipped with 488nm argon laser (BD Pharmingen) and the graphics were generated using the WinMDI 2.8 software or FlowJo 7.6 software.

2.6 Mesenchymal stem cell culture

Kidney-derived MSCs were cultured in aseptic operating conditions in MSC media (Table 2.2) supplemented with 10% (vol./vol.) FCS and 1% (vol./vol.) L-glutamine, 1% (vol./vol.) penicillin/streptomycin solution was added to the media to prevent

bacterial contamination. Once expanded *in vitro* up to passage 12, cells were stored in liquid nitrogen, as detailed in section 2.6.3.

<u>Reagent</u>	<u>Volume (ml)</u>	<u>Final concentration</u>
DMEM	450	-
FCS	45	10% (vol. /vol.)
L-glutamine	5	1% (vol. /vol.)
Penicillin/streptomycin solution	5	100U/ml penicillin + 0.1mg/ml streptomycin

Table 2.2 Quantity and final concentration of each component of mesenchymal stem cell medium for 500ml.

2.6.1 Mesenchymal stem cell trypsinisation

Trypsinisation was performed to remove the growing MSCs from the tissue culture surface once the cells became confluent. The cells could then be sub cultured. MSC culture medium was removed, since serum in the media inhibits the enzyme activity of trypsin. The cells were then washed in sterile PBS. 0.05% trypsin/0.02% EDTA was added so that all of the cells on the tissue culture surface were covered and the flask of cells incubated at 37°C for 1.5-3 min depending on the surface area of the culture vessel. The trypsin was needed to disrupt the anchorage of the cells to surface of the culture vessels and EDTA needed to chelate Ca^{2+} , which prevents calcium-dependent cell adhesion. At the end of the incubation period, the flask was tapped to maximise the detachment of cells and 4-7ml MSC medium subsequently added to prevent any further action of trypsin. The cells were then dissociated by pipetting up and down and the cell suspension then centrifuged at 400 x g for 5 min. During this time, an aliquot of the cell suspension was used for estimating the cell number present, as described in section 2.6.2. The supernatant was then removed and the cells resuspended in fresh MSC medium. Cells were seeded at the density required to ensure that approximately 70% of the tissue culture surface was covered in cells following attachment within the following 12 hr.

2.6.2 Estimation of cell number

Estimation of cell number was important for optimal cell survival and growth during culture and also for the experimental outcomes of this thesis. Cells were counted using a Neubauer haemocytometer. A cover slip was placed over the haemocytometer forming a chamber with a fixed volume of 0.1mm. Cells were retrieved from the culture vessel by trypsinisation as described in section 2.6.1. A 10µl aliquot of the cell suspension was mixed with 10µl of 0.4% trypan blue solution. Trypan blue cannot cross the membrane of viable cells and therefore selectively stains dead cells. The trypan blue stained cells were pipetted into the haemocytometer chamber and the number of live and dead cells counted in each of the four 1x1mm corner squares under a light microscope (Coolpix, MDC lens, Nikon, Japan) at x100 magnification. The total cell number per ml was estimated using the following formula:

$$\text{Cell number per ml} = \text{average cell number per } 0.1\text{mm}^3 \times \text{dilution factor (e.g 2 if cell suspension diluted 1:1 with trypan blue)} \times 10^4$$

2.6.3 Cryopreservation of mesenchymal stem cells

After MSCs had been expanded *in vitro* (cells were not expanded beyond passage 12), they were stored in 2ml cryotubes at -196°C (in liquid nitrogen), for use in subsequent experiments. MSC medium was aspirated from the culture vessel and sterile PBS was used to rinse the culture vessel and aspirated. The MSCs were then trypsinised, as detailed in 2.6.1. However, the end of the protocol was slightly different to that described, in that the cell pellet was resuspended in cryopreservant containing FCS supplemented with dimethylsulphoxide (DMSO) at a concentration of 10% v/v, as opposed to MSC medium. Cells were aliquoted into 2ml cryotubes at a density of 1×10^6 and placed in a cell freezing box containing 100% isopropanol and kept at -80°C overnight. The following day, the cryotubes were transferred to liquid nitrogen for storage.

2.6.4 Recovery and maintenance of mesenchymal stem cells

MSCs used for both *in vitro* and *in vivo* experiments were used between passages 7-12. MSCs were retrieved from liquid nitrogen and the cryotube containing the cells held underneath warm running water until a small ice pellet remained to thaw the cells

rapidly. Cells were then resuspended in MSC medium (Table 2.2) and seeded into a T25 or T75 culture vessel. MSCs were maintained in a tissue culture incubator at 37°C in an atmosphere of 5% CO₂. The medium was replaced 3x weekly and when the cells became 90-100% confluent they were sub cultured by trypsination (section 2.6.1) into a new culture vessel containing fresh MSC medium.

2.7 Islet-MSC co-culture

Preliminary experiments (detailed in Chapter Seven) utilised three culture groups (Figure 2.3) to determine whether preculturing islets with MSCs using either a direct contact co-culture system or transwell co-culture system, would help to improve islet function *in vitro*, compared to control islets cultured in the absence of MSCs. These experiments were designed to establish the method by which preculturing islets with MSCs, would be most likely to improve islet transplantation outcome in diabetic mice.

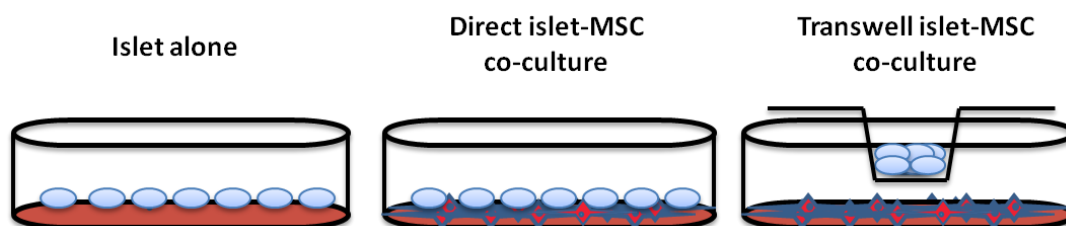


Figure 2.3 Schematic depiction of islet-MSC co-culture system. Three experimental groups were used to investigate the effect of MSCs on islet function *in vitro*. Islets alone were used as a control; islets were cultured directly with MSCs to test the relative importance of direct islet-MSC contact and a transwell islet-MSC co-culture system used to determine whether the presence of soluble MSC-secreted factors alone (in the absence of cell-cell contact) would affect islet function.

2.7.1 Direct contact islet-MSC co-culture system

Approximately 200,000 kidney-derived MSCs of passage 7-12 were seeded into Nunclon™ 35mm 6 well plates, forming a confluent monolayer of cells within 12 hr. MSCs were cultured in 3ml DMEM supplemented with 1% vol/vol penicillin/streptomycin solution (Gibco BRL, Gaithersburg, MD, USA) 10% (vol./vol.) FCS (3ml/well), L-glutamine and incubated at 37°C in a humidified atmosphere containing 5% CO₂. The medium was changed after 24 hr, with removal of non-adherent cells. 100 freshly isolated islets were then added to each well and the

medium was replaced with 3ml/well RPMI supplemented with 1% vol/vol penicillin/streptomycin solution (Gibco BRL, Gaithersburg, MD, USA) 10% (vol./vol.) FCS. Control islets (islet-alone) were cultured alone as groups of 100, in RPMI medium, in 35mm non-adherent tissue culture Petri dishes. The RPMI media was changed after two days for both culture groups and a total culture period of three days was used for all experiments. After three days direct co-culture with MSCs, the islets had formed loose attachments with the MSCs, but could be removed for *in vitro* analysis of islet function or transplantation experiments, by gently pipetting.

2.7.2 Transwell islet-MSC co-culture system

Approximately 200,000 kidney-derived MSCs of passage 7-12 were seeded into Nunclon™ 6 well plates and cultured as with the direct contact co-culture system for the first 24 hr before addition of islets. The medium was changed after 24 hr, with removal of non-adherent cells. Cell culture inserts with a semi-permeable membrane, pore size of 1.0µm (Becton Dickinson, NJ, USA) were inserted and 100 freshly isolated islets added into the inserts. The medium was replaced with RPMI supplemented with 1% vol/vol penicillin/streptomycin solution (Gibco BRL, Gaithersburg, MD, USA) 10% (vol./vol.) FCS (3 ml/well). Control islets (islet-alone) were cultured in the same way, but using 35mm non-adherent petri dishes. The RPMI media was changed after two days and a total culture period of three days was used.

2.8 Immunohistochemistry and Immunofluorescence

Immunostaining is technique that is used to visualize the localization and abundance of proteins within a preserved tissue, using antibodies which specifically recognize and bind to the protein of interest. Target antigens can be detected through either chromogenic or fluorescent means. For the majority of experiments throughout this thesis, the 3,3'-diaminobenzidine (DAB) developing system was used where the protein of interest appears as a brown end-product at the site of the target antigen, when visualized underneath a light microscope. This is due to the activity of the Horse Radish Peroxidase (HRP) enzyme, which is conjugated to streptavidin. Alternatively and particularly where dual staining for the co-localisation of two separate proteins was required; immunofluorescence was used. For this technique, the

secondary antibody (reporter) is conjugated directly to a fluorescent tag that will absorb light and emit at a specific wavelength. The fluorescence was then measured by fluorescence microscopy.

2.9 Preparation of islets for histology

2.9.1 Fixation of islets

Fixation of tissues is important to preserve the structure of the tissues and proteins present at any specific time. Formalin is a semi-reversible cross-linking reagent, which was used as a fixative throughout this thesis. For experiments using freshly isolated or cultured islets, islets were prepared for histology as follows:

Freshly isolated or cultured islets were hand-picked using a pipette from the petri dishes in which they had been counted into following isolation, or cultured in. Groups of 100-150 islets were transferred to 1.5ml eppendorf tubes and washed in 1ml PBS using a microcentrifuge and carrying out pulse spins for approximately 15 sec. The supernatant was then removed and islets fixed in 1ml of 3.7% (vol. /vol.) neutral buffered formalin for 2 hr at room temperature, before washing in PBS and adding 1ml of 70% ethanol (EtOH). Islets could be kept in 70% EtOH until a later time-point, or directly processed for paraffin wax embedding.

2.9.2 Processing of islets and paraffin-embedding

Islets were pelleted using a microcentrifuge and the 70% EtOH removed. Islets were dehydrated in graded concentrations of EtOH. 1ml of 80% EtOH with 4 drops of 1% filtered eosin was added for 5 min, followed by 95% EtOH for 5 min and then 100% EtOH for 5 min. Finally, 1ml of xylene was added for 3 min, after which the xylene was removed and 1ml 60°C paraffin wax that had been melted in a wax oven poured gently into the tube containing the islets. The islets/paraffin wax were kept in the wax oven for 4 hr, in which time the islets sank to the bottom of the tube forming a pellet, clearly visible from the pink eosin. At this point, the paraffin embedded islets (in 1.5ml microcentrifuge tubes) were transferred to 4°C, thereby allowing the wax to set. A corkscrew was used to screw into the wax most of the way down the microcentrifuge tube, but not to the point where the islets were at the bottom. 100ml

of water in a glass beaker was then boiled in a microwave and the bottom 2/3 of the eppendorf tube submerged in the boiling water for approximately 3 sec. The paraffin wax with the islets still at the tip could then be pulled out of the tube, enabling the tip of the moulded wax to be cut using a clean scalpel. The islets at the tip were then placed into a plastic mould and 60°C paraffin wax filled to the top of the template, so that the islets spread out into the same plane, and left for 30 min. This ensured that there were a lot of islets present in each paraffin section (see section 2.9.4 below). A plastic cassette was then gently placed over the top of the template and a small amount of 60°C paraffin wax added to the cassette, which was left for a further hr. The whole plastic mount (containing 5 separate mounts/blocks) was then transferred to 4°C for ≥ 2 hr, after which point the paraffin blocks were ready to section.

2.9.3 Fixation and processing of graft-bearing kidneys and pancreata

After removal of graft bearing kidneys and pancreata, these tissues were fixed in 3.7% (vol. /vol.) neutral buffered formalin for 72 hr, before washing in PBS and transferring to 70% EtOH. For graft bearing kidneys, the tissue was cut using a scalpel directly in front of the islet graft, which was visible under a dissecting microscope before processing. The tissues were then processed for paraffin embedding using a Leica TP1020 processing machine. For this, tissues were placed into uniquely labelled cassettes and then into the processing basket. The processing machine was programmed for 120 min each in the following solutions: 1) 90% IMS (IMS- 99% Industrial methylated spirit) 2) 100% IMS (3x 120 min in fresh solutions 3) IMS:Xylene 1:1 4) Xylene (2x 120 min in fresh solutions 5) Paraffin wax (1x 1 hr). The tissue was then orientated on a block using forceps to ensure that the wax was moulded in the correct orientation around the tissue, so that the tissue could be sectioned in the right plane. 60°C wax was added, followed by the cassette lid and then enough wax to cover this too. The blocks were lastly placed on a cold plate to solidify before sectioning.

2.9.4 Sectioning of paraffin embedded islets/tissue sections

5µm histological tissue sections were cut from paraffin blocks using a Leitz Wetzer microtome. The ribboned sections were then flattened out onto a few drops of 10%

EtOH placed onto a large glass slide, which was used to transfer the flattened sections into a water bath at 40°C. When the tissues were completely flat they were mounted on glass slides (VWR superfrost plus).

2.9.5 Haematoxylin and Eosin staining

Tissue sections were dewaxed and rehydrated by placing slides onto a 56°C electro thermal slide drying bench for 10 min in order to adhere tissues to the glass slides, before dewaxing and rehydrating the tissue sections in Xylene (1), Xylene (2), 100% EtOH, 95% EtOH, 70% EtOH (5 min washes each). Slides were placed into a casting frame and submerged in each of these solutions, which had been made up in glass trays, ensuring that the tissue sections were not allowed to dry out at any point (or any subsequent point during the immunostaining procedure detailed in section 2.9.6). After the last EtOH wash, slides were submerged in Mayer's hematoxylin (Sigma) for 3-5 min depending upon the intensity of staining required. After which, slides were washed in tap water for 5 min and de-differentiated using acid EtOH (0.5% HCl in 70% EtOH), by dipping the slides into this solution 4x. Slides were then washed under tap water for 5 min and submerged in 1% aqueous eosin solution (Rymond A Lamb) for 5 min, before washing in water again. Sections were dehydrated in the following solutions 70% EtOH, 95% EtOH, 100% EtOH, Xylene (1), Xylene (2) for 5 min each. Sections were mounted using DPX mountant (Sigma-Aldrich) and were then ready for visualisation by light microscopy and analysis (see section 2.9.8)

2.9.6 Method for immunostaining

At the start of the immunostaining procedure, tissue sections were dewaxed and rehydrated as above (section 2.9.5). After rehydrating the tissue sections, they were incubated in 3 % v/v hydrogen peroxide (30% stock solution, Sigma-Aldrich, diluted 1:10 in deionised water), which was made up in a coplin jar, for 10 min. This was to block endogenous peroxidase activity. Tissue sections were then washed in tap water for 5 min.

For CD34 and α -SMA staining; antigen retrieval was carried out in order to restore the immunoreactivity of these proteins. The tissue sections were treated with 1mmol/l

citric acid solution (8ml of citric acid stock solution in 800ml deionised water, pH to 6.0 with 5M NaOH), which was prepared in a pressure cooker. The solution was heated in a microwave with the lid resting on the cooker at full power for 10 min or until boiling, after which the slides in a casting frame were submerged in the buffer. At this point the lid with pressure valve was placed on the pressure cooker and 2 min timed after full pressurization (a distinct 'hissing' sound can be heard and the pressure valve elevated). After 2 min, the tissue sections were allowed to cool for approximately 15 min while the cooker was de-pressurized. The lid was then removed and sections were rinsed with tap water for 15 min to remove any residual citric acid solution. Antigen retrieval was not necessary for the detection of any of the proteins other than CD34 and α -SMA, but did not interfere with the immunostaining protocol for other proteins, if it had to be carried on whole slides with multiple tissue sections, on which immunostaining consecutive sections was necessary. The remainder of the staining procedure was the same for all antibodies used and is as follows:

Slides were placed onto a humidified chamber and kept here at room temperature for all incubation steps unless stated otherwise. Tissue sections were circled with a 'PAP' pen (Invitrogen life technologies) to prevent solutions from running off the slide or merging into adjacent sections. Endogenous biotin was blocked using a biotin blocking system (Dako Cytomation). Tissue sections were incubated with avidin for 10 min (80 μ l of each reagent was sufficient to cover tissue sections) and then washed in Tween-buffered Saline (TBS) (see Table 2.3), before incubating with biotin for a further 10 min. Non-specific antibody binding was then blocked by incubating tissue sections for 10 min in 2% BSA blocking buffer (see Table 2.4). The appropriate primary antibody was applied to slides for 1 hr at room temperature or overnight at 4°C (see Table 2.5 for appropriate dilutions). Omission of the primary antibody was used as a negative control. Tissue sections were washed in TBS, after which the tissues were incubated with either biotin- or fluorochrome-conjugated secondary antibodies (see Table 2.5) depending upon the detection system used.

For immunofluorescence, sections were counterstained with DAPI (1:1000, Jackson Immunolaboratories) and mounted using fluorescent mounting medium (Dako). At

which point, the slides were ready to be visualised and photographed, using a Nikon digital sight DS QIMC camera and NIS-Elements BR 3.0 software.

For protocols using the DAB detection system, tissue sections were incubated with streptavidin HRP (Invitrogen life technologies) for 30 min at room temperature, washed in TBS and developed for 1-10 min in DAB solution, gently agitating slides intermittently during this time period. The chromogenic response between the HRP enzyme and DAB substrate was monitored under a light microscope for optimal colour intensity. The reaction was stopped by rinsing slides with water for 5 min. Sections were counterstained using hematoxylin and differentiation was carried out using acid ethanol (0.5% HCl in 70% EtOH).

Sections were dehydrated in the following solutions 70% EtOH, 95% EtOH, 100% EtOH, Xylene (1), Xylene (2) for 5 min each and mounted using DPX mountant (Sigma-Aldrich).

2.9.7 Reagents

3.7% formaldehyde

37% formaldehyde solution (Sigma-Aldrich) was diluted 1 in 10 in PBS to obtain a 3.7% formaldehyde solution.

10x Tween Buffered Saline (TBS) (for 1L)

<u>Reagent</u>	<u>g/L</u>
Sodium Chloride	88.0
Trizma® Base	60.0

Table 2.3 Quantity of each component of TBS for 1L. The volume was made up to 1L with deionised (DI) water and stored at room temperature.

1% BSA Blocking Buffer/ antibody diluent

<u>Reagent</u>	<u>Volume/weight</u>
DI water	178ml
Bovine Serum Albumin	2g
10% sodium azide	2ml
10x TBS	20ml

Table 2.4 Volume of each component of blocking buffer for 200ml. All reagents were dissolved fully and 5-10ml aliquots stored at -20°C until use.

Citrate buffer

The citric acid stock solution (1M) was made by dissolving 105g citric acid in 500ml deionised water. This was then stored at room temperature.

<u>Antibodies</u>	<u>Dilution for DAB detection system</u>	<u>Dilution for immunofluorescence</u>	<u>Specificity</u>
Polyclonal guinea pig anti-Insulin (Dako)	1:1000	1:100	Primary Ab
Monoclonal mouse anti-Glucagon (Sigma) *	1:1000	1:100	Primary Ab
Monoclonal rat anti-Somatostatin (AbCam)	1:50		Primary Ab
Monoclonal rat anti-CD34 (AbD Serotec)	1:200		Primary Ab
Monoclonal rat anti-CD31 (Dianova)	1:80		Primary Ab
Monoclonal rabbit anti-α-SMA (AbCam)	1:500	1:100	Primary Ab
Goat biotinylated Anti-Guinea Pig IgG (H+L) (Jackson Immunolaboratories)		1:200	Secondary Ab
Rabbit biotinylated anti-rat IgG (H+L) (Vector Laboratories)		1:200	Secondary Ab
Goat anti-guinea pig Texas Red (Jackson Immunolaboratories)		1:40	Secondary Ab
Donkey anti-mouse Dylight (Jackson Immunolaboratories)		1:40	Secondary Ab

Table 2.5 List of antibodies with their dilutions for immunohistochemistry and immunofluorescence, as well as their specificity. *A biotinylated link universal (part of the Dako LSAB+ system-HRP kit), was used in place of the secondary Ab for immunostaining with glucagon and α -SMA antibodies. Abbreviations: Ab, antibody. H, heavy chain. L, light chain.

2.9.8 Evaluation of Vascular Density

All immunostaining of endothelial cells (ECs) was done using the DAB detection system. Therefore, images were taken with an Olympus BX40 light microscope and Q imaging micropublisher 5.0 RTV camera, using Q capture Pro (v.5.1) software. Adobe Photoshop Elements 4.0 and Image J computer software (<http://rsbweb.nih.gov/ij/>) were used for calculating the islet area and counting the number of ECs (hematoxylin stained nuclei with surrounding brown cytoplasm showing positive staining) within this area. The number of ECs was counted at a

magnification of x400. More detailed descriptions for the evaluation of vascular density are given in individual results chapters.

2.10 Measurements of insulin secretion *in vitro*

2.10.1 Measurement of insulin content

To determine the insulin content of islets, 10 islets in triplicate were picked for each condition, washed twice in PBS, before adding 200µl of acidified ethanol (absolute ethanol: deionised water: concentrated HCL in the ratio of 52:17:1). Samples were sonicated on ice, for 3x 5 second pulses, output 10-14 and insulin extracted overnight at 4°C. Samples were then stored at -20°C until a later time point for analysis. Each sample was diluted with borate buffer (see Table 2.6) to an appropriate concentration for the detection limits of the radioimmunoassay. The insulin content of each sample was then measured by radioimmunoassay (section 2.10.4).

2.10.2 Measurement of basal and glucose stimulated insulin secretion

The effects of MSC co-culture were assessed using static incubations of islets. Islets were pre-incubated for 2 hr in RPMI containing low glucose (2mmol/l). To measure basal and glucose stimulated insulin secretion, groups of three islets were then transferred to 1.5ml eppendorf tubes and incubated in 300µl of 2mmol/l or 20mmol/l Gey and Gey buffer (see Table 2.7) respectively, at 37°C for 1 hr. At the end of the incubation period, the islets were centrifuged at 2,000 rpm, for 2 min, at 4°C. 200µl of the supernatant was then removed and added to 800µl of borate buffer in 1.5ml eppendorf tubes. The insulin content of the incubation medium was then assessed by RIA.

2.10.3 Reagents

Borate Buffer

For 2L:

<u>Reagent</u>	<u>Amount for 2L (g)</u>	<u>Final concentration (mmol/l)</u>
Boric acid	16.5	133
NaOH	5.4	67.5
EDTA	7.4	10

Table 2.6 Weight and concentration of each component of borate buffer. All reagents were dissolved in approximately 1.8L of deionised water, before adjusting the pH to 8.0 with concentrated HCl. The volume was then made up to 2L with deionised water and BSA (100% w/v) added before storing at 4°C ready to use.

Gey and Gey Buffer 2x Stock Solution

For 1L:

<u>Reagent</u>	<u>Amount (g)</u>	<u>Concentration (mmol/l)</u>
NaCl	6.50	222.0
NaHCO ₃	2.27	54.0
KCl	0.37	9.92
MgCl ₂ .6H ₂ O	0.21	2.06
Na ₂ HP0 ₄ .2H ₂ O	0.09	1.00
MgSO ₄ .7H ₂ O	0.07	0.56
KH ₂ PO ₄	0.03	0.44

Table 2.7 Weight and concentration of each component of Gey and Gey Buffer (Gey and Gey 1936). All reagents were dissolved in approximately 800ml of deionised water and the volume made up to 1L with deionised water.

Gey and Gey buffer working solution (2 and 20mmol/l glucose)

250ml of 2x Gey and Gey buffer stock solution was added to 250ml deionised water to make the working solution. 180mg D-glucose (MW-180) was added to make the glucose concentration of the buffer 2mmol/l. The pH was adjusted to 7.4 using CO₂. 1ml of 1M CaCl₂ and 250mg bovine serum albumin (BSA) were added. To make a

20mmol/l glucose working solution, 64.8mg D-glucose was added to 20ml of 2mmol/l Gey and Gey solution.

2.10.4 Insulin radioimmunoassay

The molecular basis of the radioimmunoassay technique is based on the recognition of an antigen by a specific antibody (ie anti-insulin). The technique was initially developed to measure insulin (Yalow and Berson, 1960) and has been used in this thesis for measuring the insulin content of isolated/cultured islets, islet grafts and the amount of insulin secreted into the media during static incubation experiments.

A standard curve was prepared with known concentrations of insulin covering the range over which the assay is sensitive (0.08, 0.16, 0.32, 0.64, 1.25, 2.5, 5 and 10ng/ml). This is done by carrying out serial dilutions of a 10ng/ml insulin stock, diluted in borate buffer. Reference tubes were set up to determine maximum binding, non-specific binding and totals. Antibody, tracer and standards were added to LP3 tubes as outlined in Table 2.8. The standards were assayed in triplicate and samples in duplicate. The tubes were covered in parafilm and left to equilibrate at 4°C for 48 – 72 hr.

	<u>Buffer (μl)</u>	<u>Antibody (μl)</u>	<u>Tracer (μl)</u>	<u>Standard (μl)</u>	<u>Sample (μl)</u>
Non-specific binding (NSB)	200		100		
Maximum binding (Bo)	100	100	100		
Totals (T)			100		
Standards		100	100	100	
Samples		100	100		100

Table 2.8 Preparation of standards, reference tubes and samples for RIA.

Following the incubation period, 1ml of precipitant (15% polyethylene glycol, PEG) was added to all tubes, except the totals, and the tubes were then centrifuged at 3000 x g for 15 min at 4°C. The supernatant was then aspirated and a γ -counter used to measure the radioactivity of the pellets (as counts per minute (cpm)).

2.10.5 Reagents

PBS

95.5g Dulbecco's phosphate buffered saline was dissolved in 9L of deionised water. 3g sodium azide (0.03% w/v) was added and the volume made up to 10L with deionised water and the solution kept at room temperature.

30% PEG

600g polyethylene glycol (PEG, MW 6000) was dissolved in 1L of deionised water and the volume made up to 2L. This solution was stored at 4°C.

Precipitant

The 30% PEG solution was diluted 1:1 with PBS containing γ -globulin (final concentration 1mg/ml) and Tween 20 (final concentration 0.5 μ l/ml).

Antibody

Anti-insulin antibody raised against bovine insulin in Hartley Guinea pigs (Jones and Howell, 1989) was used at a final concentration of 1:60,000.

¹²⁵I-Insulin

The tracer was diluted in borate buffer at a concentration which would obtain approximately 10,000cpm per assay tube. The amount of tracer needed was determined for each individual assay, due to radioisotopic decay.

2.11 Analysis of gene expression

2.11.1 Isolation of mRNA from mouse islets

Total RNA was extracted using the RNeasy mini kit (Qiagen, Crawley, UK). The kit is a column-based extraction kit. RNA binds to the silica-based membrane, with the microspin technology efficiently removing any contaminants, such as unwanted DNA in the cell lysate. The RNeasy mini kit also enriches for mRNA based on size, with most mRNAs being greater than 200 nucleotides, whilst selectively excluding other RNAs (rRNA and tRNA), which are usually less than 200 nucleotides.

Briefly, 150 islets were hand-picked under a dissecting microscope, resuspended in PBS and transferred to RNase/DNase free micro centrifuge tubes. 350µl RNA lysis buffer (RLT buffer in the kit), supplemented with β -mecaptoethanol at a dilution of 1:100, was added to each tube and the tube vortexed to ensure adequate lysis, before storing at -80°C until required for the extraction process. RNA extraction from both freshly isolated and cultured islets was carried out at the same time. Fresh lysis buffer was made up at each time point.

RNA extraction was carried out according the manufacturers guidelines. An equal volume of 70% EtoH was added to the lysate and a Gilson pipette used to mix. The suspension was then added to the spin column, which was centrifuged at 13000 x g for 1 min. 350µl of wash buffer (buffer RW1 in the kit) was then added to the column and the flow through discarded. The column was then centrifuged again at 13000 x g for 1 min.

An additional DNase digestion step was carried out using the RNase-free DNase set (Qiagen) in order to remove any small amounts of DNA that may still be present. After this, the column was washed again with 350µl of wash buffer, before transferring the column to a new 2ml collection tube. The column was then washed twice with RPE buffer, with a longer 2 min centrifuge spin for the second wash ensuring that the RNeasy silica-gel membrane was dried and that there was no residual EtOH remaining in the column. To prevent any transfer of RPE buffer, the column was then washed again for 1 min at 13000 x g. The column was finally transferred to a new 1.5ml collection tube for the elution stage. 30µl of RNase free water was applied directly to the column, which was centrifuged for 1 min at 13000 x g to elute the RNA. This elute was then re-applied to the column to obtain a higher RNA concentration. The RNA samples were then quantified before storing at -80°C for subsequent cDNA synthesis.

2.11.2 Measurements of RNA concentration and purity

After extracting total RNA the concentration and purity of each sample was determined using a spectrophotometer (NanoDrop ND-1000; NanoDrop

Technologies, Wilmington, USA). Measurements were made at an absorbance of 260nm (A_{260}) after blanking with 1.5µl of RNase free water.

2.11.3 cDNA synthesis

In order to perform quantitative PCR, the extracted single strand RNA must be reverse transcribed into double stranded cDNA. The SuperScript™ II room temperature enzyme (Invitrogen life technologies Life Technologies) was used to synthesis cDNA according to the manufacturer's guidelines.

2.11.4 Method for cDNA synthesis by reverse transcription

All RNA samples were thawed on ice and kept on a cool block for the cDNA synthesis in order to maintain RNA integrity. The volume of RNA was calculated based on the concentration of RNA as determined from the nanodrop readings; for each room temperature reaction 100ng of total RNA was used. For each sample, the volume was made up to 10µl with RNase free water (Sigma). 1µl of random primers (100ng) and 1µl of dNTP Mix (10mmol/l) each was added to each sample in a microcentrifuge tube. This mixture was then heated at 65°C for 5 min to melt the secondary structure of the template and immediately chilled on ice for 5 min in order to prevent the secondary structure from re-forming. A master mix was prepared according to Table 2.9 and 8µl added to each sample. Each sample was then incubated at 42°C for 50 min to ensure that the SuperScript™ II room temperature was activated and had time to synthesise the cDNA. The enzyme was then inactivated by heating at 70°C for 15 min. The newly synthesised cDNA was diluted 1:5 with molecular biology grade water and stored at -20°C for subsequent quantification of gene expression by Quantitative PCR.

Reagent	1x sample
5x First-Strand buffer	4 μ l
DTT (0.1M)	2 μ l
RNAasin ® (40U/μl) Plus RNase inhibitor	1 μ l
SuperScript™ II RT	1 μ l (200 units)

Table 2.9 Components and volumes for each component of the cDNA synthesis reaction.

2.12 Real- time quantitative PCR

2.12.1 Analysis of gene expression by RT-qPCR

Analysis of gene expression was determined by quantitative PCR using a Real-Time Light Cycler® LC480 PCR machine (Roche Diagnostics, UK) and FastStart DNA Master SYBR Green I kit (Roche Diagnostics, UK).

All PCR reactions were carried out using the same amplification conditions, except for the annealing temperature, which varied depending upon the primer pair used, as specified in Table 2.10. The amplification conditions were as follows: 5 min at 94°C followed by 40 repeated cycles of: 30 sec at 94°C (denaturation step), 1 min at the specific annealing temperature for each primer pair and 1 min at 72°C (elongation step). The fluorescence was acquired during this final elongation step.

Gene	Primer sequence	Product size (bp)	Annealing temp (°C)
CD34	F: TTG ACT TCT GCA ACC ACG GA R:TAGATGGCAGGCTGGACTTC	300	60
CD31	F: GAGCCCAATCACGTTTCAGTT R: TCCTTCCTGCTTCTTGCTAGC	181	61
β-actin	F: ATGAAGTGTGACGTTGACATCCT R:CCTAGAAGCATTGCGGTGCACGATG	300	58

Table 2.10 RT-PCR primer sequences, product sizes and melting temperatures.

Abbreviations: bp, base pair. F, forward. R, reverse.

For the use of new sets of primers, the PCR product needs to be isolated and purified to make standards with a known concentration of the target DNA, so that the amount of amplified DNA (gene of interest) in subsequent assays can be determined. This was done by purifying the DNA sequences for each PCR product on an agarose gel and subsequently carrying out serial dilutions of DNA standards as explained briefly below.

2.12.2 Gel electrophoresis

Reagents

10X Tris borate EDTA(TBE) buffer (for 1L):

<u>Reagent</u>	<u>Amount (g)/L</u>
Tris- (hydroxymethyl) methylamine	109.3
Boric acid	55.65
EDTA	9.31

Table 2.11 Quantity of each component of 10X TBE buffer for 1L.

The volume was made up to 1L with deionised water and stored at RT. 1X TBE buffer was then used as the running buffer for the electrophoresis.

Agarose gels

1.8% agarose gels were made by dissolving 0.9g of electrophoresis grade agarose in 50ml of 1X TBE buffer. The mixture was heated in a microwave for approximately 1 min with intermittent swirling to ensure that all of the agarose was completely dissolved and taking care to avoid the formation of air bubbles. This solution was cooled for approximately 5 min and 0.5µl 10ng/ml ethidium bromide added. This solution was then poured into a gel tray and allowed to set. The gel was submerged in 1X TBE buffer. 10µl of the PCR product was mixed with 2µl of 6 X loading buffer and the samples were then run alongside a molecular weight marker (1Kb DNA ladder, Promega) for approximately 40 min at 70 volts. The gel slice containing the DNA sequence as visualized under UV light, using a transilluminator was then excised using a clean scalpel blade and transferred to DNase, RNase free tubes.

2.12.3 Purification of DNA from Agarose gels

DNA was extracted from the gel slice using a QIAquick gel extraction kit (Qiagen), according to manufacturer's instructions. Briefly, the weight of the gel slice was measured, so that three volumes of the kit QG buffer could be added. The slice was then incubated at 50°C for 10 min with occasional vortexing. One volume of isopropanol was added to obtain a greater yield of DNA. The sample was then added to the QIAquick spin column, which was centrifuged at 1000 rpm, 1 min). During this step, DNA binds to the column and impurities such as salts, enzymes, unincorporated nucleotides, agarose and ethidium bromide are removed. 500µl of buffer QG was then used to wash the column again for the removal of any remaining agarose. The column was then washed with 150µl of PE buffer for the removal of any residual salts. The flow-through was discarded and the column centrifuged for the removal of any remaining ethanol. Finally, the DNA was eluted by adding 30µl of EB buffer to the column. Gel extracted DNA was diluted and serially diluted 10-fold in molecular biology grade water to produce seven standards of known concentration (ranging from 10^9 – 10^3 copies). Purified DNA was stored at -20°C.

2.12.4 Real- time quantitative PCR method

Forward and reverse oligonucleotide primers complimentary to the gene of interest are detailed in Table 2.10. The lyophilised primers specific to the gene of interest were made up to a stock concentration of 100µM in molecular biology grade water. They were then diluted to a working concentration of 10µM. PCR reactions were set up in 20µl reactions (as detailed in Table 2.12) using either the target gene of interest cDNA or the standard cDNA , as recommended in the manufacturers instruction for using the Light Cycler® 480 SYBR green I master mix with Light Cycler® 480 multiwell plate 384 (Roche).

<u>Reagent</u>	<u>Volume (μl)</u>	<u>Final concentration</u>
Forward primer (10μM)	1	500nM
Reverse primer (10μM)	1	500nM
Molecular biology grade H₂O	11	-
Master mix (2x)	5	1x
cDNA/H₂O	2	-
Total	20	

Table 2.12 Preparation of PCR reaction mix.

Standards were processed together with the cDNA of interest. For each reaction, a negative control was included, to ensure that there was no contaminating genomic DNA present (a total RNA blank). qRT-PCR was performed as described previously in section 2.12.1. For all reactions, a standard curve to the house keeping gene (β -actin) was set up using the same method, to allow for normalisation of the target cDNA sequence. Melt curve analysis was performed to ensure that only a single product had been amplified and results were presented as a percentage decline relative to the absolute quantification of the gene product in fresh islets.

2.13 Statistical Analysis

Data are expressed as means \pm SEM unless stated otherwise. When two groups were compared, Student's *t* test was used. When more than two groups were compared, one-way ANOVA was used. For experiments in which more than one variable was being compared, two-way ANOVA was used. If the differences between groups in the ANOVA reached statistical significance, individual groups were compared using Bonferroni's multiple comparison test, or Dunn's multiple comparison test if individual groups were being compared to the same control group. Results were considered significant at $p < 0.05$. All statistics were carried out using sigma plot 11.0.

3 Chapter Three - The effect of islet culture on revascularisation and transplantation outcome

3.1 Introduction

Transplantation of islets of Langerhans can be used successfully as a treatment for Type 1 Diabetes Mellitus (T1DM) in humans and is also able to reverse hyperglycaemia in rodent models of diabetes. Vast improvements in the human islet transplantation field were made with the development of the Edmonton protocol during the 1990's (Shapiro et al., 2000). Despite the initial success of this study, follow-up investigations have shown that there is a progressive decline in graft function, with less than ten percent of patients remaining insulin independent after five years (Ryan et al., 2005a). Although some centres have had successful outcomes with single-donor transplantations (Hering et al., 2004, Posselt et al., 2010, Matsumoto, 2011), the large majority of islet transplant recipients require multiple donors (Shapiro et al., 2000, Goss et al., 2002, Barshes et al., 2004), with a similar donor to recipient ratio observed in rodent models of diabetes. Thus, there are evidently problems associated with suboptimal function (Ahn et al., 2007, Lau et al., 2007, Mattsson et al., 2004) and survival (Davalli et al., 1996, Biarnes et al., 2002, Miao et al., 2006) of the transplanted islets, limiting the widespread application of islet transplantation as a therapy for patients with T1DM.

Islets of Langerhans are highly organised aggregates of endocrine cells with a rich vascularisation and specific organisation of endocrine cells (Orci and Unger, 1975), which ensures that blood glucose is controlled optimally. Islets in the endogenous pancreas typically have a dense glomerular-like vascular structure (Vetterlein et al., 1987), which ensures their supply with oxygen and rapid distribution of hormones to target tissues (Brunnicardi et al., 1996, Lammert et al., 2003), with every β -cell in contact with at least one capillary endothelial cell (EC) (Bonner-Weir and Orci, 1982, Brunnicardi et al., 1996). Additionally ECs provide the β -cells with a basement membrane, creating a 'vascular niche', thereby providing a microenvironment which stimulates their endocrine function (Nikolova et al., 2006). The islet isolation

procedure disrupts these vascular connections and therefore the transplanted islets are avascular during the immediate post transplantation period (Menger et al., 1989, Miao et al., 2006). The transplanted islets therefore have to function in a hypoxic microenvironment (Miao et al., 2006) until the revascularisation process is completed.

Hypoxia contributes to non-specific inflammatory events by up-regulating gene products such as tissue factor (TF) and Monocyte Chemoattractant Protein (MCP) within the transplanted islets (Johansson et al., 2003). Whilst MCP is known for its potent chemoattractant activity for monocytes, TF is the major trigger for the IBMIR, with both factors exerting a negative influence on engraftment and transplantation outcome (Johansson et al., 2005a). Hypoxia can also upregulate NF κ B, which is in general regarded as an anti-apoptotic factor, but under certain conditions, can have pro-apoptotic functions (Ortis et al., 2008) and activate genes mediating inflammation (Hu et al., 2005). Moreover, when hypoxia persists, death programmes are activated leading to the extensive β -cell apoptosis and necrosis, which has been reported to occur during this time (Biarnes et al., 2002, Davalli et al., 1996, Miao et al., 2006). The revascularisation process is known to be completed within 7-14 days post transplantation (Menger et al., 1992, Mendola et al., 1994, Merchant et al., 1997). However, there is accumulating evidence suggesting that this process is insufficient, regardless of the implantation site used (Mattsson et al., 2002b, Mattsson et al., 2003, Olsson and Carlsson, 2005, Lau and Carlsson, 2009), which results in suboptimal islet oxygen tension (Carlsson et al., 1998) and compromised long term graft function and survival.

One of the criteria of the Edmonton protocol was the use of fresh, as opposed to cultured islets. Previous human islet transplantation trials had commonly utilised cultured islets, largely due to the practicalities associated, but these former trials were less successful. More recently, islet transplant centres worldwide have reverted to using cultured islets (Goss et al., 2002, Shapiro et al., 2003, Hering et al., 2004, Froud et al., 2005, Kin et al., 2008), largely for logistical reasons. Practical benefits of culturing human islets include the time this enables to initiate time-dependent immunosuppressive regimens, transport islets to the transplant centre and carry out safety/quality control testing of islet preparations (Kin et al., 2008).

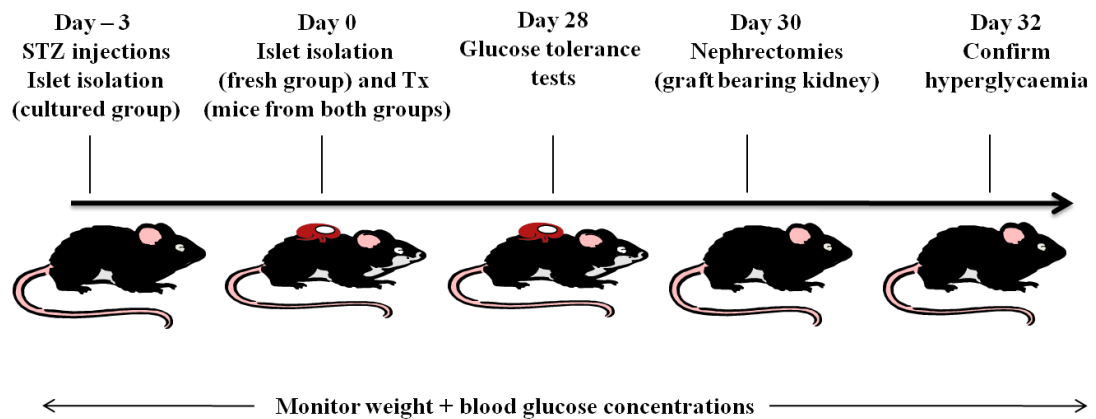
A number of experimental studies in rodents have shown that fresh islets produce superior transplantation outcomes to cultured islets (King et al., 2005, Olsson and Carlsson, 2005). This has partially been attributed to an increased rate (Nyqvist et al., 2011) or overall extent of islet revascularisation (Olsson and Carlsson, 2005), with an important correlation between islet EC density and β -cell function established in a number of studies (Lai et al., 2005, Johansson et al., 2009c). Furthermore, it is clear that a substantial loss of human islet material occurs during the pre-transplant culture period (Kin et al., 2008). Given the current shortage of donor islet material available for human islet transplantation, there is considerable interest in determining the importance of islet culture for transplantation outcomes, to ensure that the donor islets are utilised as best is possible.

The aims of this study were: 1) to determine the extent to which culture affects islet composition, in terms of endocrine cell number and vascularisation and to correlate this with graft function; and 2) to quantify islet graft EC density during the immediate post transplantation period and also at two and four weeks after transplantation; a time point when the revascularisation process is known to be completed.

3.2 Methods

3.2.1 Experimental protocol to test the efficacy of fresh and cultured islet grafts in a syngeneic minimal islet mass model

Mice in both transplant groups (fresh and cultured islet graft recipients) were made diabetic by a single high-dose i.p. streptozotocin injection (180 mg/kg) three days prior to the transplantation, as detailed in section 2.3.1. This study was designed so that mice in both transplant groups received islet transplants on the same day to ensure that any difference in STZ potency/severity of diabetes could be controlled for. This also allowed for IPGTTs to be carried out at exactly the same time point post-transplantation for both transplant groups. Mice were allocated to receive either fresh or cultured islets ensuring that the average starting blood glucose (day 0) was similar between transplant groups. A minimal mass of 200 islets was used based on previous studies (King et al., 2005).



Schematic representation of the experimental protocol used to investigate the effects of islet culture on graft function in STZ-diabetic mice.

3.2.2 Endothelial cell staining

CD34 has previously been shown to be a suitable EC marker for both pancreas and kidney tissue in C57BL/6 mice (Mattsson et al., 2002a). Therefore, CD34 antibodies were used to characterize the abundance and localisation of ECs in endogenous pancreatic islets, freshly isolated, cultured, and transplanted islets. The lectin *Bandeiraea simplicifolia* (BS-1) has also been used in a number of studies investigating islet revascularisation, due to its selectivity for ECs in mice (Laitinen, 1987). BS-1 was therefore used as an additional marker to investigate the abundance and localisation of ECs within freshly isolated and cultured islets. The method for CD34 immunostaining was the same throughout this thesis and is described in section 2.9.6.

3.2.3 Staining with the lectin *Bandeiraea simplicifolia*

Lectins are carbohydrate-binding proteins and can be used in a similar way to antibodies, in that they show selective, reversible binding to specific configurations of terminal saccharides associated with cell surfaces, thus selectively binding to certain cell types (Sharon and Lis, 1989). Specifically, *Bandeiraea simplicifolia* BS-1 binds to D-galactosyl residues, which are present on the surface of ECs (Laitinen, 1987). Tissue sections were dewaxed and rehydrated as detailed in section 2.9.5. After which, tissues were pre-treated with neuraminidase type X (Sigma) to remove

blocking sialic acid residues. Neuraminidase was made up as a stock solution of 1U/ml using sodium acetate buffer and stored at 4°C. To obtain a working concentration of 0.5U/ml, the neuraminidase stock solution was diluted 1:1 using sodium acetate buffer (see Table 3.1) and applied to sections at a concentration of 0.5U/ml for 2 hr at 37°C.

The slides were washed in cold deionised H₂O prior to following the same blocking stages detailed in section 2.9.6. The biotinylated BS-1 lectin (1:100) was applied to tissue sections for 1hr at room temperature after which slides were washed in TBS. Emission of the biotin-conjugated BS-1 lectin was used as a negative control. Streptavidin-HRP was applied to the slides for 30 min and the procedure was then the same as that described for all other antibodies using chromogenic detection of target antigens (see section 2.9.6).

Reagents

Sodium acetate buffer

<u>Reagent</u>	<u>Mass (g)</u>	<u>Final concentration</u> <u>(mmol/l)</u>
Sodium acetate anhydrous	0.820	100
Calcium Chloride	0.029	2

Table 3.1 Mass and final concentration of each component of sodium acetate buffer for 100ml.

All reagents were dissolved in 100ml of deionised water and the pH adjusted to 5.0 at 37°C using 1M HCL.

3.2.4 Evaluation of Vascular Density

All immunostaining of ECs was done using the 3,3'-diaminobenzidine (DAB) developing system. Therefore, images were taken using an Olympus BX40 light microscope and Q imaging micropublisher 5.0 RTV camera, using Q capture Pro (v.5.1) software. Adobe Photoshop Elements 4.0 and Image J computer software (<http://rsbweb.nih.gov/ij/>) were used for calculating the islet area and counting the

number of ECs within this area. The number of ECs was counted at a magnification of x400.

3.2.4.1 Endogenous pancreatic islets, freshly isolated and cultured islets

The vascular density was defined as the number of intraislet islet ECs (CD34+ or BS-1+ cells) per islet area. For each time point (freshly isolated islets, one day, two day and three day cultured islets) ≥ 15 islets were analyzed from each mouse (n=4). The same analysis was used for endogenous islets present in pancreas tissue sections from non-diabetic non-transplanted mice.

3.2.4.2 Transplanted islets

For the initial experiments investigating the vascularisation of transplanted islets in normoglycaemic mice (in comparison with endogenous islets) 7-12 tissue sections throughout the entire islet graft from each mouse were evaluated. After which ≥ 5 tissue sections throughout the islet graft were evaluated in each animal. For the analysis of islet graft vascularisation in diabetic mice, the grafts immunostained and evaluated were from the same experimental groups as those shown for graft function. The tissue was sectioned so that the analysis was done throughout the graft to ensure that the mean value was obtained for the vascular density of transplanted islets and that any regional differences were taken into account. The number of ECs was counted at a magnification of x400 and only ECs present in the endocrine components of the graft were included in the analysis. The area of the graft was determined using Image J software and the vascular density (number of ECs/mm²) was determined. The demarcation of the islet graft was taken as the area of endocrine tissue in between the renal parenchyma and the kidney capsule and the presence of an islet graft was also confirmed by insulin staining of consecutive islet graft sections for the detection of β -cells.

3.3 Results

3.3.1 Graft function

Transplantation of freshly isolated islets produced superior transplantation outcomes to islet grafts consisting of three day cultured islets (Figure 3.1). Blood glucose concentrations were monitored for one month after transplantation. Average blood glucose concentrations were significantly lower at 14 and 21 days post transplantation

in mice transplanted with fresh islets, compared to those transplanted with cultured islets (Figure 3.1a). At one month post transplantation, all mice transplanted with fresh islets were cured, compared with only 40 percent of mice transplanted with cultured islets (Figure 3.1b). Only mice with a starting blood glucose concentration of 20-25mmol/l were included in these results to ensure that the severity of diabetes was similar between transplant groups. There were no differences in the starting weight of mice in each transplant group (22.82 ± 1.18 and 22.26 ± 0.80 g, fresh vs cultured islet recipients respectively, $p = 0.70$, $n=5$, Student's t test). At 28 days post transplantation, IPGTTs were carried out in all cured mice, as well as age-matched non-transplanted non-diabetic controls. Glucose tolerance was similar between mice transplanted with freshly isolated islets and non-diabetic non-transplanted controls (Figure 3.1c). However, mice transplanted with fresh islets had a significantly lower weight at the time of the IPGTT than control mice (21.16 ± 0.81 vs 24.0 ± 0.67 g, $n = 4-5$, $p = 0.04$, Student's t test). Statistical analysis was not carried out for the mice transplanted with cultured islets, as only two mice had cured in this group; however the glucose tolerance profile appeared to be similar to that of mice transplanted with fresh islets, as well as controls. At one month post transplantation all cured recipients (cure was defined as two consecutive non-fasting blood glucose levels <11.1 mmol/l, without reversion to diabetes at any subsequent date) were nephrectomised to confirm that the STZ-diabetic recipients were cured due to islet graft function, as opposed to regeneration of β -cells in the endogenous pancreas. All mice in both transplant groups reverted to hyperglycaemia within two days (Figure 3.1d).

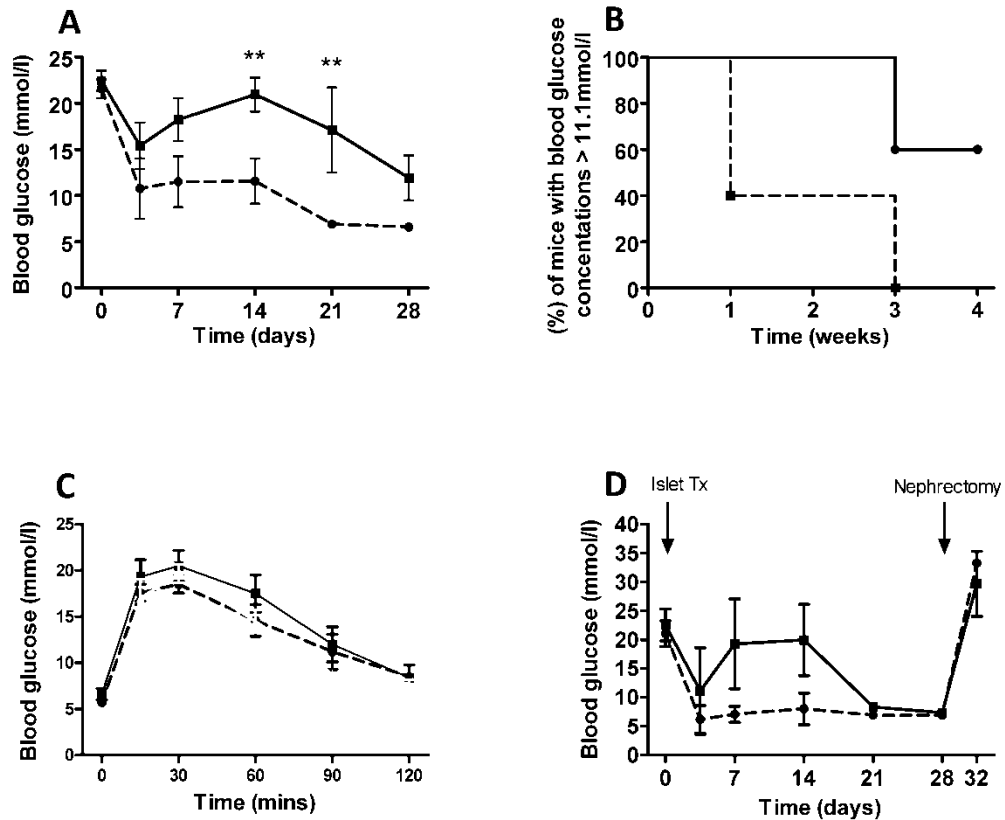


Figure 3.1 Graft function **A.** Blood glucose concentrations of mice transplanted with freshly isolated islets (dashed line) or three day cultured islets (continuous line); ** $p < 0.01$ vs mice transplanted with freshly isolated islets (RM ANOVA with Bonferroni post hoc test, $n=5$ for both transplant groups). **B.** Percentage of mice remaining diabetic (blood glucose concentration >11.1 mmol/l) after transplantation as in A, $p = 0.02$ Kaplan–Meier, $n=5$ for both transplant groups. **C.** IPGTTs in all cured mice at 28 days post transplantation and age-matched non-diabetic non-transplanted controls, $p > 0.05$, two-way RM ANOVA, $n=4-5$, freshly isolated islets (dashed black line), three day cultured islets (continuous black line), control mice (continuous grey line). **D.** Blood glucose concentrations of cured mice only after islet transplantation (day 0) and after removal of the graft bearing kidney at one month post transplantation ($n=2-3$). **A, C** Data expressed as means \pm SEM, **D.** Data expressed as means \pm SD.

Immunostaining of fresh (Figure 3.2a) and cultured (Figure 3.2b) islet grafts with insulin antibodies confirmed the presence of viable β -cells beneath the renal capsule at one month post transplantation, with no major differences in graft morphology observed.

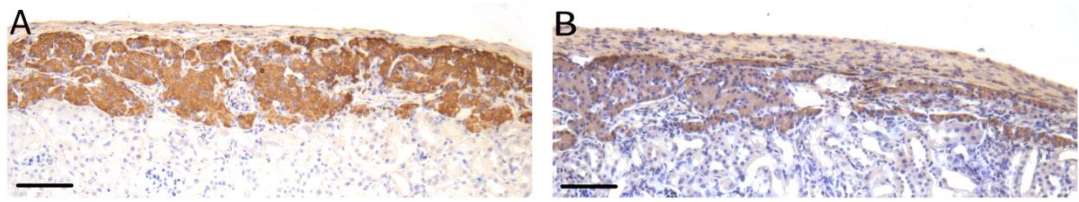


Figure 3.2 Graft morphology. Immunostaining of islet grafts consisting of freshly isolated (a) and three day cultured islets (b) with insulin antibodies, at one month post transplantation to STZ-diabetic recipients, original magnification x100, scale bars are 100µm.

3.3.2 Endocrine cell number in freshly isolated and cultured islets

Insulin and glucagon antibodies were used to immunostain β - and α -cells respectively, in freshly isolated and three day cultured islets. As expected, the majority of islet cells stained positively with insulin antibodies, with unlabelled cells at the periphery for both fresh (Figure 3.3a) and cultured (Figure 3.3b) islets. The number of β - and α -cells per islet section was quantified in order to determine whether a three day culture period would affect their number. The number of β -cells per islet section was reduced after three days culture (Figure 3.3c); while the number of β -cells per islet area was increased (Figure 3.3d). Islet area declined from 16015 ± 1701 to $5993 \pm 601\mu\text{m}^2$ during the three day culture period ($p = <0.0001$, Student's t test, $n=4$).

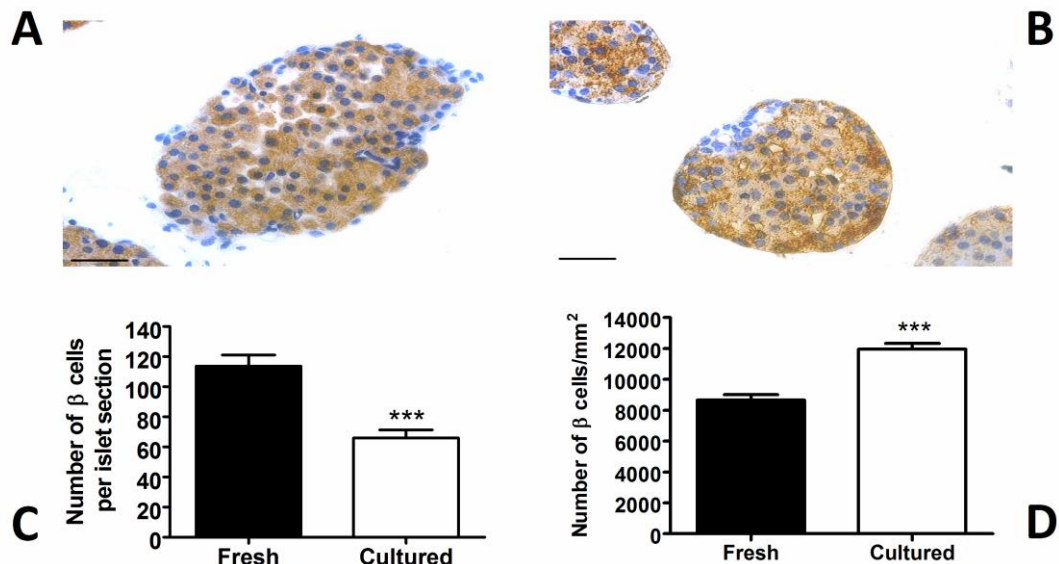


Figure 3.3 β -cell number after three days culture. Immunostaining of β -cells with insulin antibodies in freshly isolated (a) and three day cultured (b) ICR mouse islets, original magnification x400, scale bars are 25µm. **C, D** β -cell number per islet section and number of β -cells per islet area, in same islets, *** $p < 0.0001$ vs freshly isolated islets, Student's t test, $n=4$. Fresh (70 islets counted), three day cultured (85 islets counted) from four mice.

The unlabelled cells in the islet mantle appeared to be mostly α -cells in both fresh (Figure 3.4a) and cultured islets (Figure 3.4b), as shown by glucagon immunostaining. The number of α -cells was unaffected by three days culture (Figure 3.4c), as was the number of α -cells per islet area (Figure 3.4d). Although the islet area decreased during the three day culture period, the total number of cells (all nuclei within the islet section) per islet area increased (7466 ± 393 and 12870 ± 430 cells, fresh and cultured islets respectively, $p < 0.001$, $n=4$), as expected with 80-90 percent of cells being insulin-positive, thus the isolated islets became more compacted during culture.

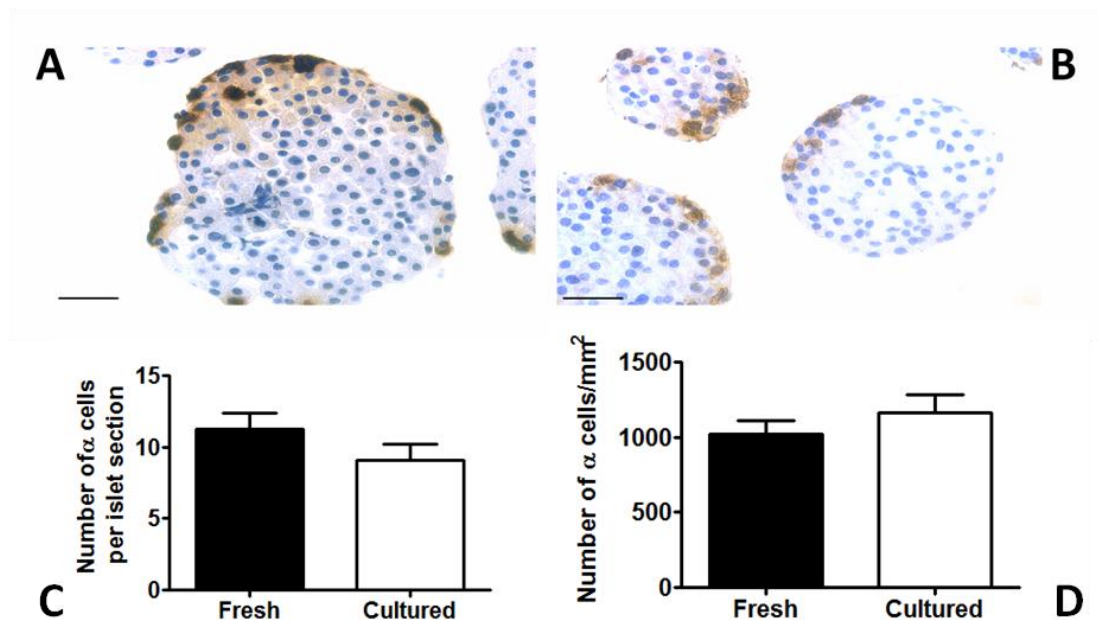


Figure 3.4 Alpha cell number after three days culture. Immunostaining of α -cells with glucagon antibodies in freshly isolated (a) and three day cultured (b) ICR mouse islets, original magnification x400, scale bars are 25 μ m. C, D. α -cell number per islet section and number of α -cells per islet area, in same islets, $p < 0.05$ vs freshly isolated islets, Student's t test, $n=4$. Fresh (76 islets counted), three day cultured (70 islets counted) from four mice.

3.3.3 Distribution of endocrine cells in freshly isolated and cultured islets

The distribution of β - and α -cells was similar in both fresh and cultured islets, with insulin positive β -cells located in the islet core, surrounded by an incomplete rim of glucagon positive α -cells (Figure 3.5).

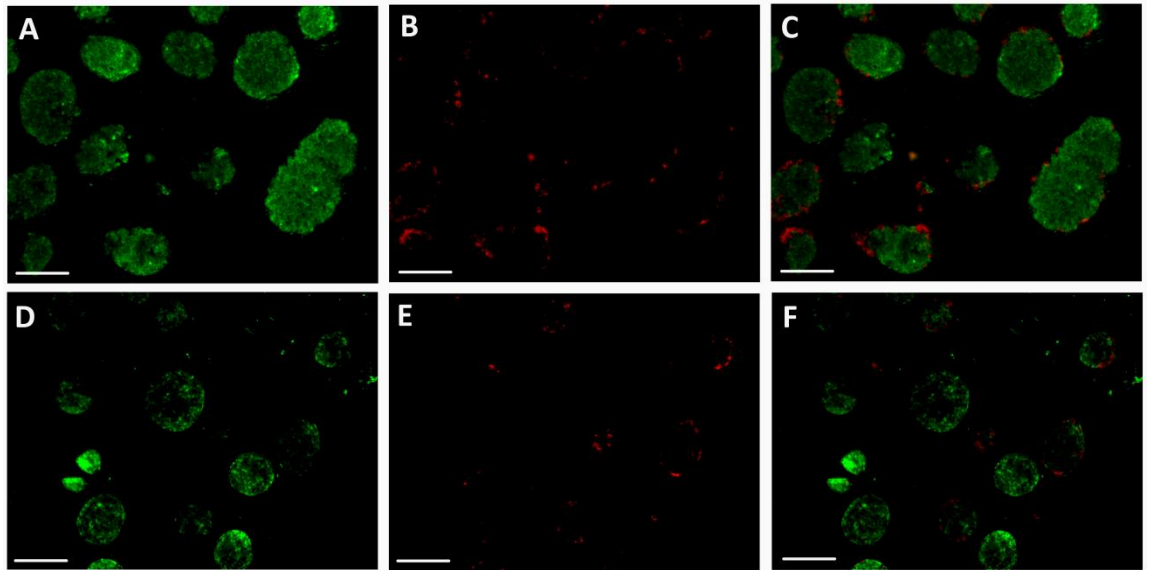


Figure 3.5 Distribution of endocrine cells. Immunostaining of β -cells with insulin antibodies (green) and α -cells with glucagon antibodies (red), in freshly isolated islets (upper panel) and three day cultured islets (lower panel), original magnification x200, scale bars are 100 μ m.

3.3.4 Rapid and significant decline of CD34-positive endothelial cells *in vitro*

The loss of intraislet ECs during a three day culture period is both extensive and very rapid. CD34 antibodies were used to stain microvascular ECs in freshly isolated islets, as well as short term cultured islets (1-3 days post isolation). The distribution of CD34+ ECs in freshly isolated islets (Figure 3.6a) was similar to that of endogenous pancreatic islets, with ECs distributed throughout the islet. CD34+ ECs were also distributed throughout the one and two day cultured islets (Figure 3.6b and c), however their number was clearly reduced. After three days, there were very few CD34+ ECs remaining (Figure 6d). Quantification of CD34+ ECs, indicated that the vascular density (number of ECs/mm²) of short term cultured islets was 52%, 24% and 5% that of freshly isolated islets, for one day, two day and three day cultured islets respectively (Figure 3.6e). After one and two weeks culture we were unable to detect any CD34+ ECs at all, indicating that there is no expansion of donor ECs during a longer culture period. As well as the rapid and dramatic loss of ECs during a three day culture period, there was also a significant decline in islet area ($13,062 \pm 1467$ vs 5338 ± 578 μ m², $p = 0.003$, $n=4$), confirming previous findings of islets analysed for endocrine cell number, which were from different islet isolations.

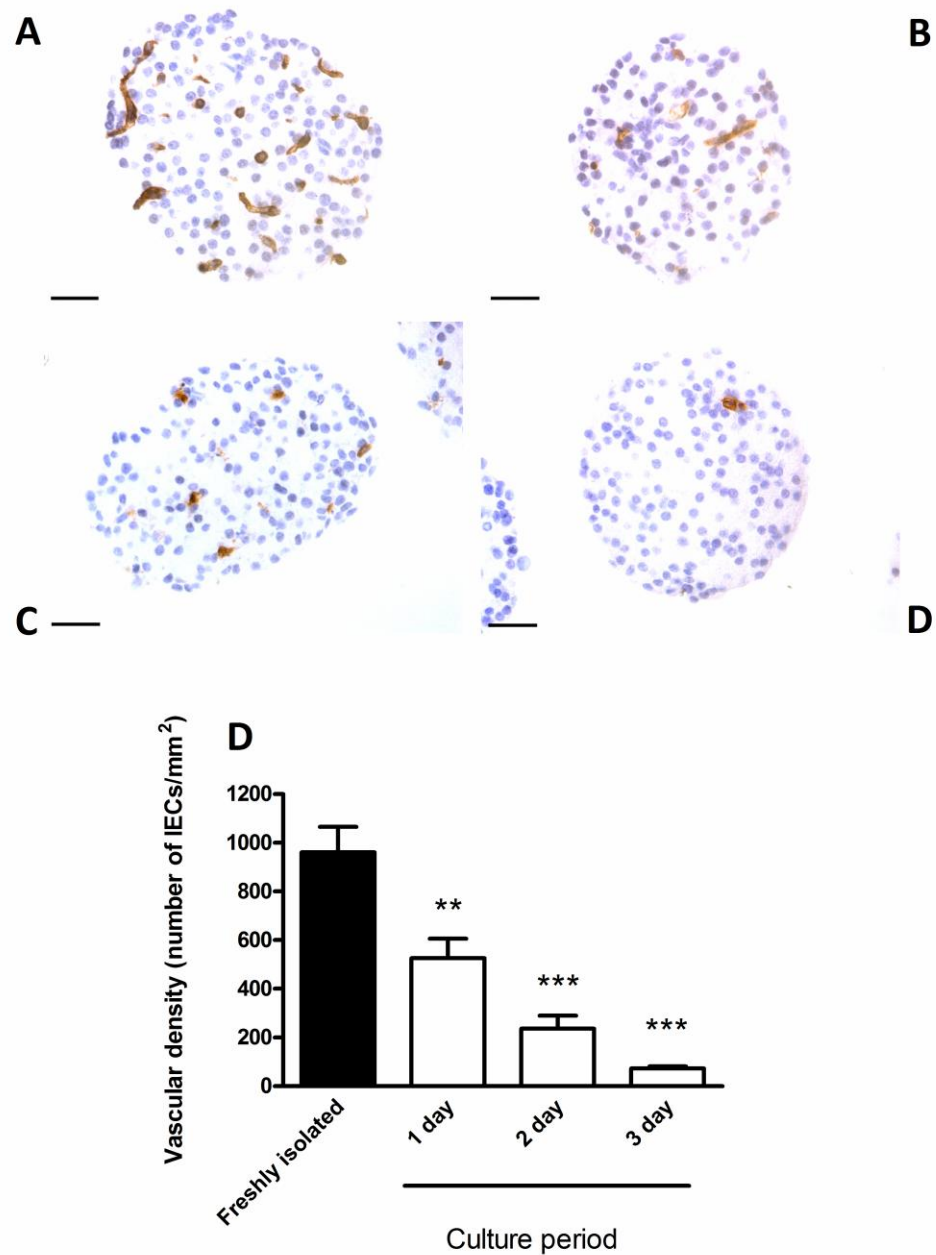


Figure 3.6 Rapid decline of CD34+ ECs during a three day culture period. Immunostaining of ECs with CD34 antibodies in freshly isolated (a), one day (b), two day (c) and three day cultured (d) ICR mouse islets, original magnification x400, scale bars are 25µm. **E.** Vascular density of fresh and cultured islets; ** $p < 0.01$, *** $p < 0.001$ vs freshly isolated islets (ANOVA and Dunnett's post hoc test; all groups of cultured islets compared to freshly isolated islets), fresh (74 islets counted), one day (128 islets counted), two day (80 islets counted) and three day (77 islets counted) cultured islets from four mice.

3.3.5 Decline of BS-1-positive ECs *in vitro*

A second microvascular EC marker; the lectin *Bandeiraea simplicifolia* (BS-1), was used to assess further the extent to which ECs are lost after three days in culture. The

distribution of BS-1+ ECs in freshly isolated islets (Figure 3.7a) was similar to that shown using CD34 as an EC marker. BS-1+ECs were also distributed throughout three day cultured islets. Again, their number was dramatically reduced, albeit not to the same extent as that shown for CD34+ ECs (Figure 3.7b). Analysis of BS-1+ EC number showed a significant reduction in the vascularisation of three day cultured islets (Figure 3.7c), confirming the dramatic loss of ECs during short term culture. Despite this, staining for microvascular ECs with BS-1 indicated that the vascular density of three day cultured islets was 31 percent that of freshly isolated islets; indicating that there may be a subset of ECs that express BS-1, but not CD34, or that BS-1 is not completely selective for ECs.

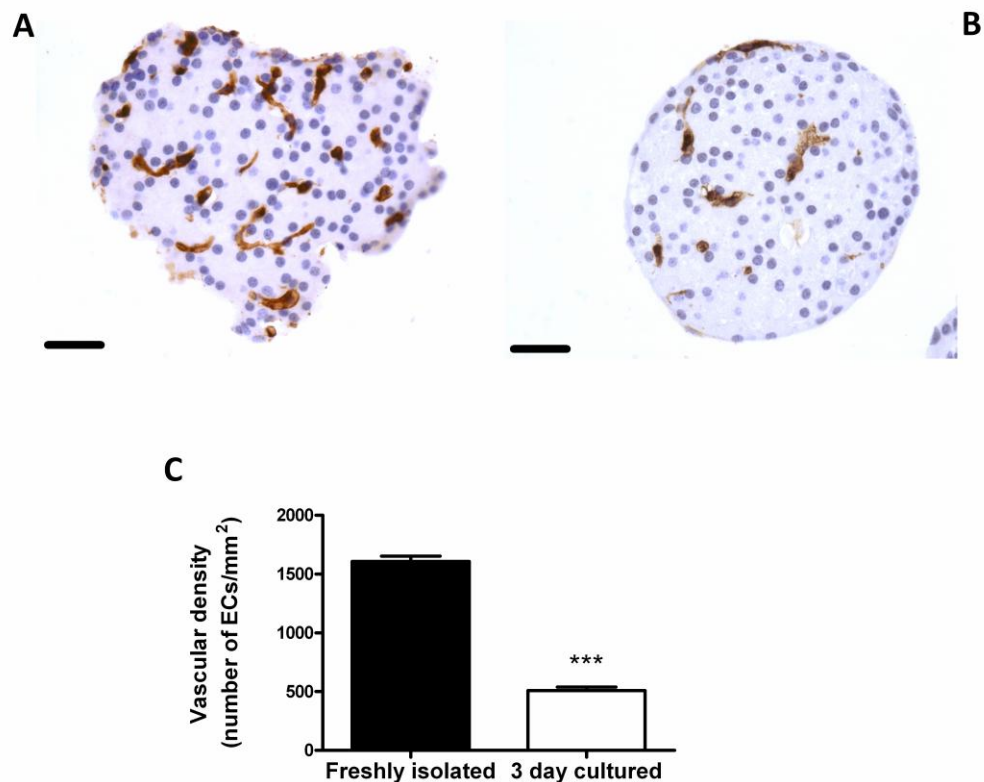


Figure 3.7 Rapid decline in BS-1 positive ECs after a three day culture period. Staining of ECs with the lectin *Bandeiraea simplicifolia* in fresh (a) and three day cultured (b) ICR mouse islets, original magnification x400, scale bars are 25μm. C. Vascular density of fresh and cultured islets, n=4, *** $p < 0.001$ vs freshly isolated islets, Students t test, fresh (88 islet counted), and three day cultured (74 islets counted) islets from four mice.

3.3.6 Quantification of CD34 mRNA expression in fresh and cultured islets

Melting curve analysis confirmed the presence of a single PCR product for both fresh and cultured islets (Figure 3.8a). Cultured islets showed a significant decline in the expression of CD34 mRNA in comparison to freshly isolated islets (Figure 3.8b). The percentage decline in CD34 mRNA expression was comparable to that of CD34 at the protein level (shown in Figure 3.6), indicating that the decline in CD34 protein is correlated to the expression of CD34 mRNA.

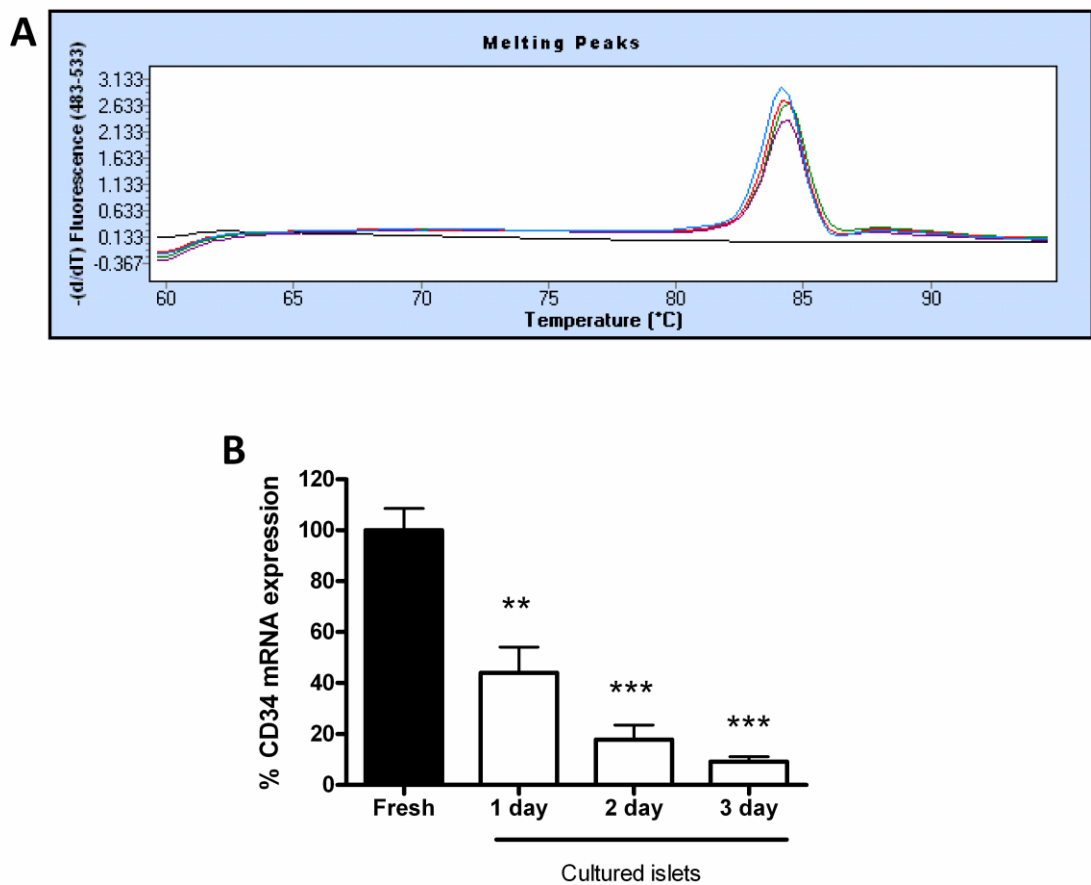


Figure 3.8 Decline of CD34 mRNA expression during a three day culture period. Groups of 150 mouse islets (n=3) were used and RNA isolated from extracts. CD34 mRNA expression was measured by quantitative PCR. The results are expressed relative to β -actin mRNA in the same extracts. **A.** Melting curve analysis from quantitative LightCycler™ PCR. Forward and reverse primers were used to amplify a 300bp product from mouse cDNA (Blue line: fresh islets, red line: one day cultured islets, green line: two day cultured islets and purple line: three day cultured islets). Total RNA (tRNA, black line) was used as a negative control. Results are from one experiment typical of three experiments. **B.** Percentage decline in CD34 mRNA expression, ** $p < 0.05$, *** $p < 0.001$ (One-Way ANOVA with Dunnett's post hoc test; all groups of cultured islets compared to freshly isolated islets, n=3).

3.3.7 Quantification of CD31 mRNA expression in fresh and cultured islets

It has previously been suggested that CD34 may be down regulated in proliferating ECs (Fina et al., 1990). Other studies have used a mixture of CD34/CD31 antibodies (double staining), with the proliferation marker Ki67 to identify proliferating ECs (Hillen et al., 2006). CD31 has been shown to be expressed on proliferating ECs (Bencini et al., 1993), as well as cultured islet ECs (Nygqvist et al., 2005) and ECs which have been isolated from islets and then cultured (Johansson et al., 2009a). Therefore, we used CD31 as an additional EC marker to determine whether there was a similar change in the expression of both CD34 and CD31 EC markers after culture. This is important to address the question as to whether ECs de-differentiate during culture so that they no longer express CD34, or whether other EC markers are lost to a similar extent. CD31 mRNA was quantified in the same islet extracts as used for the quantification of CD34 mRNA. Melting curve analysis confirmed the presence of a single PCR product for fresh and cultured islets (Figure 3.9a). Cultured islets expressed significantly lower levels of CD31 mRNA than freshly isolated islets, showing a similarly rapid and significant decline to that of CD34 mRNA (Figure 3.9b).

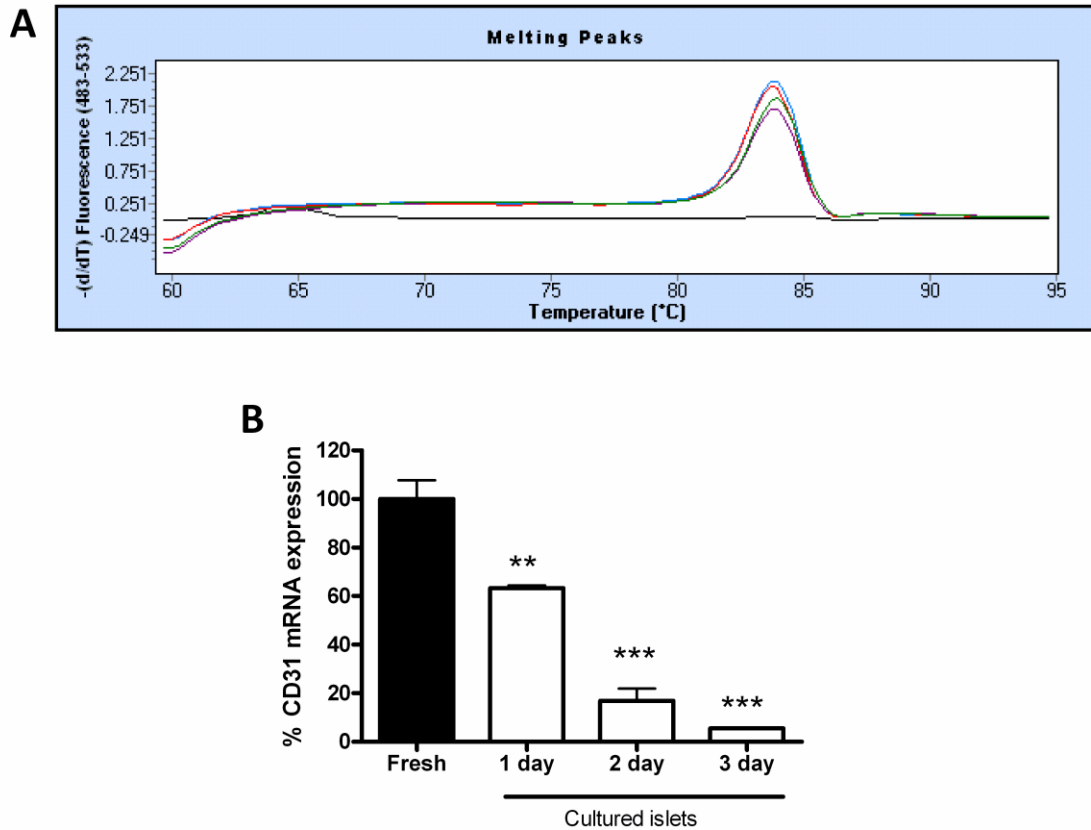


Figure 3.9 Decline of CD31 mRNA expression during a three day culture period. Groups of 150 mouse islets ($n=3$) were used and RNA isolated from extracts. CD31 mRNA expression was measured by quantitative PCR. The results are expressed relative to β -actin mRNA in the same extracts. **A.** Melting curve analysis from quantitative LightCyclerTM PCR. Forward and reverse primers were used to amplify a 181bp product from mouse cDNA (Blue line: fresh islets, red line: one day cultured islets, green line: two day cultured islets and purple line: three day cultured islets). Total RNA (tRNA black line) was used as a negative control. Results are from one experiment typical of three experiments. **B.** Percentage decline in CD31 mRNA expression ** $p < 0.05$, *** $p < 0.001$ (One-Way ANOVA with Dunnett's post hoc test; all groups of cultured islets compared to freshly isolated islets $n=3$).

3.3.8 Vascular density of islets transplanted into normoglycaemic recipients

Microvascular endothelium in endogenous pancreatic islets and islets transplanted underneath the kidney capsule of normoglycaemic C57BL/6 recipients were stained with CD34 antibodies. Endogenous pancreatic islets were richly vascularised, with ECs distributed equally throughout the islet core and mantle. The majority of unstained nuclei, presumably representing endocrine cells were in contact with at least one EC (Figure 3.10a). At three days post transplantation, the endocrine tissue within the grafts was essentially avascular with very few CD34+ ECs detected in any of the graft material analysed (Figure 3.10b). At two weeks after transplantation, CD34+

ECs could be detected within the endocrine tissue, although their localisation was often predominantly at the periphery of the engrafted islets (Figure 3.10c). The vascular density of the transplanted islets at two weeks, increased relative to that at three days. However, the revascularisation process is clearly suboptimal, as shown by the decreased vascular density in comparison to islets in the endogenous pancreas (Figure 3.10d).

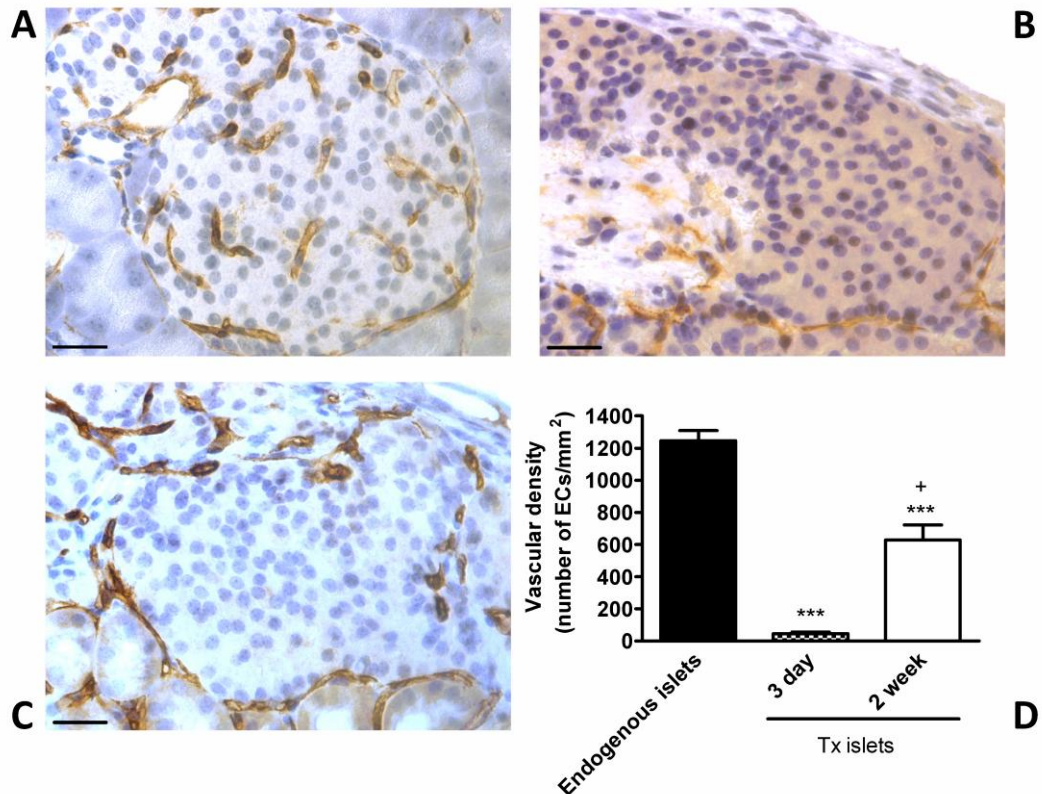


Figure 3.10 Vascular density of endogenous pancreatic islets and islets transplanted underneath the kidney capsule of normoglycaemic mice. Immunostaining of ECs with CD34 antibodies in endogenous pancreatic C57BL/6 islets (a) and freshly isolated islets transplanted underneath the kidney capsule at three days post transplantation (b) and two weeks post transplantation (c), original magnification x400, scale bars are 25µm. **D.** Vascular density of endogenous pancreatic islets (black bar) and fresh islets transplanted beneath the kidney capsule then retrieved at three days (black and white checked bar) and two weeks (white bar) post transplantation, n=3-4, *** $p < 0.001$ when compared with endogenous islets, + $p < 0.001$ when compared with three day transplanted islets, One-Way ANOVA with Bonferroni post hoc test.

3.3.9 Vascular density of islets transplanted into hyperglycaemic recipients

CD34 antibodies were used to immunostain microvascular endothelium in one month grafts consisting of freshly isolated (Figure 3.11a) and three day cultured islets (Figure 3.11b), transplanted underneath the kidney capsule of STZ-diabetic recipients.

In both transplant groups, there were areas of endocrine tissue that were devoid of ECs. The vascular density of the endocrine tissue was significantly reduced in islet grafts consisting of cultured islets, in comparison with fresh islet grafts (Figure 3.11c)

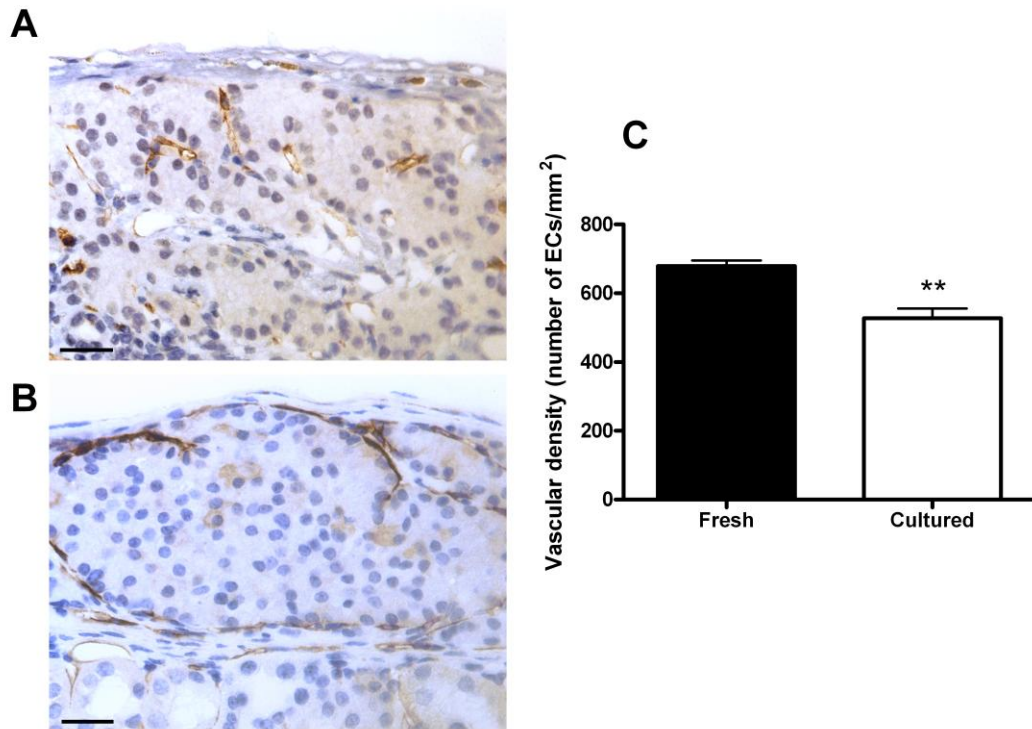


Figure 3.11 Vascular density of islets transplanted underneath the kidney capsule of diabetic mice at one month. CD34 immunostaining of islet grafts consisting of freshly isolated (a) or three day cultured islets (b) at one month post transplantation, original magnification x400, scale bars are 25µm. C. Vascular density of endocrine components in one month grafts consisting of 200 freshly isolated islets (black bar) or 200 three day cultured islets (white bar), ** $p < 0.05$ vs fresh islet grafts, Student's t test, $n = 4-6$ animals per group.

3.4 Discussion

A syngenic minimal islet mass model was used to address the importance of islet culture in terms of islet graft function and engraftment. Consistent with a number of previous studies, these experiments confirmed the superiority of fresh islets in term of both glycaemic control (King et al., 2005, Olsson and Carlsson, 2005, Takahashi et al., 2009) and vascular engraftment (Olsson and Carlsson, 2005). There are likely to be multiple factors accounting for the improved transplantation outcome with fresh islets. It is well established that the islet isolation procedure disrupts the islet vasculature and therefore the islets are dependent on their oxygen supply by diffusion during the subsequent culture period, which particularly threatens the survival of

centrally located β -cells during the subsequent culture period (MacGregor et al., 2006). A number of reports have indicated the detrimental effects of islet culture on both β -cell (Wang and Rosenberg, 1999, Keymeulen et al., 2006, Ichii et al., 2007, Kin et al., 2008) and EC survival (Parr et al., 1980, Nyqvist et al., 2005, Olsson et al., 2006). Under normal culture conditions, the islets are poorly oxygenated, with a drop in oxygen tension from the surface of the culture medium ($\sim 150\text{mmHg}$) to the vicinity of the islets ($\sim 25\text{mmHg}$). This is in addition to the islet internal pO_2 (partial pressure of oxygen) gradients in which centrally located β -cells are less well oxygenated than the peripheral cells (Lau et al., 2009), which leads to hypoxia-induced central necrosis (Vasir et al., 1998, Olsson et al., 2006).

Consistent with previous reports (Wang and Rosenberg, 1999), this study showed that β -cell number declined during culture, indicating that the improved glycaemic control seen in recipients of fresh islets may be partially explained by a greater initially transplanted β -cell mass. Indeed a number of clinical studies have also emphasised the importance of the initially transplanted β -cell number for improved long term transplantation outcomes (Warnock and Rajotte, 1988, Kaufman et al., 1990, Tobin et al., 1993).

The maintenance of α -cell number during culture is of interest as their presence is thought to have an important and beneficial influence on β -cell function *in vitro* (Carvell et al., 2007). Furthermore, the presence of α -cells within islet grafts has been shown to result in improved long term metabolic outcome in diabetic recipients (Keymeulen et al., 1996). The specific loss of β -cells *in vitro* observed in this study is indicative that there is a preferential loss of centrally located β -cells, as has previously been demonstrated and is reported to be particularly prevalent in larger islets (Vasir et al., 1998, Olsson et al., 2006), where the oxygen diffusion distance is greater. Although it is clear that a proportion of α -cells survive the isolation procedure and culture period; the typical rim of α -cells (Sujatha et al., 2004) in the rodent islet mantle appears to be incomplete in both fresh and cultured islets. This observation is in accordance with Huang and co-authors, supporting the suggestion that there is a loss of peripheral α -cells, as a result of the isolation procedure itself (Morini et al., 2006, el-Naggar et al., 1993), as opposed to the proceeding culture period. Notably

and in accordance with Moritz and colleagues (Moritz et al., 2002), it was observed that some of the freshly isolated islets had an uneven surface, indicating the loss of the peri-insular basement membrane, due to the enzymatic isolation process (Rosenberg et al., 1999). Thus, the peripheral localisation of α -cells appears to make them vulnerable to mechanical and enzymatic stresses during the isolation procedure, while hypoxic stresses during the culture period are particularly detrimental to centrally located β -cells (Olsson et al., 2006).

The substantial loss of β -cells can at least partially account for the decline in islet area observed in this study. Additionally, there was also a dramatic loss of intraislet ECs during culture, in accordance with previous reports (Parr et al., 1980, Nyqvist et al., 2005, Olsson et al., 2006). It is possible that the increased number of β -cells per islet area observed in cultured islets compared to fresh may be related to this loss of ECs and the subsequent collapsing of remnant vascular spaces, thus leading to the compaction of cultured islets, as suggested by Ahn and co-authors (Ahn et al., 2007). The number of α -cells per islet area was not altered by the culture process, which is likely to be because α -cells only made up ten percent of the total cells present in islet sections of both fresh and cultured islets. Additionally, their peripheral location is likely to make them less susceptible to hypoxic stresses, as indicated by studies showing that significant pO_2 gradients develop within isolated islets (Lau et al., 2009). Notably, there is a growing body of evidence suggesting that small islets are superior in terms of survival and function *in vitro*, but also for improved transplantation outcome (MacGregor et al., 2006, Kampf et al., 2006, Lehmann et al., 2007). Thus, there appears to be a complex interplay between the importance of islet size, but also the total β -cell mass transplanted, as well as additional factors related to the culture period, such as EC survival.

The survival of intraislet ECs is important for several reasons. Firstly, there is evidence to suggest that the presence of donor islet ECs is beneficial for an increased rate (Nyqvist et al., 2011) or overall extent of islet revascularisation *in vivo* (Nyqvist et al., 2005, Brissova et al., 2004), which in turn correlates with improved graft function in terms of glycaemic control and curative capacity (Lai et al., 2005, Johansson et al., 2009c). Additionally, a number of recent studies have indicated the

importance of ECs in terms of paracrine effects on adjacent β -cells (Linn et al., 2003b, Johansson et al., 2009a). Reciprocal interactions take place between β -cells and ECs during islet development (Lammert et al., 2001, Konstantinova and Lammert, 2004); further indicating the importance of these two cell types for each other's optimal function. A number of separate studies have demonstrated that islet-EC co-culture has beneficial effects on islet function *in vitro* (Song et al., 2009), with EC- secreted trophic factors enhancing glucose stimulated insulin secretion, total insulin content and the rate of glucose oxidation (Johansson et al., 2009a).

Intraislet ECs express typical cell adhesion molecules, such as V-CAM 1, VE-cadherin, von Willebrand factor, thrombomodulin, CD34, PE-CAM (CD31), endoglin (CD105) and ICAM-1 (Mattsson et al., 2002a, Favaro et al., 2005). However, ECs are phenotypically heterogeneous and show tremendous plasticity when subjected to different microenvironments. Previous studies have indicated that intraislet islet ECs are lost and/or de-differentiate in culture (Parr et al., 1980, Nyqvist et al., 2005, Olsson et al., 2006). Three separate EC markers were utilised for the *in vitro* studies in this study to determine whether ECs are lost to a similar extent in our hands, before carrying out any future interventions for enhancing EC survival.

Immunohistological analysis of fresh and cultured islets using both CD34 and BS-1 as EC markers, indicated that the loss of intraislet ECs is both rapid and extensive. This is in agreement with previous studies indicating that the density of BS-1-positive ECs in cultured islets is only fifteen percent that of endogenous pancreatic islets (Olsson et al., 2006). Quantitative PCR confirmed the dramatic loss of CD34+ ECs at the mRNA, as well as protein level and additionally showed the same percentage decline in CD31+ ECs, in accordance with Nyqvist and colleagues, who used immunohistochemistry to show the dramatic loss of CD31+ ECs during culture (Nyqvist et al., 2005). Thus, it seems most likely that a large majority of intraislet ECs do not survive the short term culture period.

A number of studies have indicated the importance of trophic support from the surrounding non-endocrine cells of the pancreas for islet cell survival (Dor et al., 2004, Street et al., 2004). The islet isolation procedure leads to the disruption of

normal interactions that take place between endocrine and non-endocrine cells, as well as extracellular matrix (ECM) components. This leads to a loss of both structural integrity and function of cultured islets and eventually apoptotic cell death (Rosenberg et al., 1999, Thomas et al., 1999, Bosco et al., 2000). Thus, it might be expected that the survival of both endocrine cells and ECs, would be better *in vivo* than in standard culture conditions, where islets lack the trophic support of any surrounding three dimensional tissue stroma. However, the current study does not provide evidence to support this hypothesis. Having confirmed the loss of intra-islet ECs *in vitro*, using CD34 as an additional microvascular EC marker to those previously reported by others, it was subsequently demonstrated that the same loss of EC phenotype occurred at three days post isolation *in vivo*. The finding of a virtually avascular phenotype in islets during the immediate post transplantation period is in accordance with other studies (Mendola et al., 1994, Miao et al., 2006) and supports previous reports that islet revascularisation does not begin until 2-4 days post transplantation (Menger et al., 1989, Vajkoczy et al., 1995, Linn et al., 2003b). It is important to note that a wide range of factors are likely to contribute to islet EC survival and/or death both *in vitro* and *in vivo*.

Hypoxic exposure induces a series of genes, which are transcriptionally regulated by hypoxia-inducible factor 1 alpha (HIF-1 α), all of which enable cells to adapt to low oxygen concentrations (Moritz et al., 2002). Hypoxia can regulate the expression of angiogenic factors, such as hepatocyte growth factor (HGF), which are up regulated in islets cultured in hypoxic conditions (Vasir et al., 2000). Culture of islets under hypoxic conditions has also been shown to reduce the expression of the pro-angiogenic factor basic fibroblast growth factor (bFGF) and although another proangiogenic factor; vascular endothelial growth factor (VEGF) was upregulated in the same islets, the signal was not potent enough to prevent decay of CD31 expression in the same islets (Lai et al., 2005). Thus, Lai and colleagues have suggested that islets remain angiogenically inactive during the immediate post transplantation period, because of the reduction of ECs by hypoxia. Hypoxia also has an important influence on β -cells and has been shown to regulate genes involved cell survival pathways (Hochachka et al., 1996), however if it persists cell death will occur, as shown through the co-localisation of HIF-1 α and activated caspase-3 in central regions of

islets cultured in hypoxic conditions. Similarly HIF-1 α expression has been shown to be highly expressed in transplanted islets at one, three and seven days post transplantation, with a decline in its expression at 14 days coinciding with the completion of the revascularisation process (Miao et al., 2006). The exact role of HIF-1 α is controversial, with some studies indicating that it may play a protective role enabling cells to adapt to metabolically low oxygen concentration (Ryan et al., 1998, Zhong et al., 1999). Whilst other studies have indicated that HIF-1 α has a proapoptotic role activating genes involved in cell death (Palmer et al., 1998, Bruick, 2000). Thus, the role of hypoxia is complex; whilst being an important initiator of the angiogenic process (Dionne et al., 1993), hypoxia has also been shown to kill islet ECs (Lai et al., 2005). The initiation of angiogenesis also requires the release of angiogenic factors from the transplanted islets themselves (Carlsson et al., 1998). Notably, the balance between angiogenic and angiostatic factors (Carmeliet, 2003) will ultimately determine the extent of islet vascularisation *in vivo* (Johansson et al., 2009b). Inflammatory stresses are also important determinants of both β -cell and EC survival, as well being important stimuli for the angiogenic process (Linn et al., 2006).

The peripheral localisation of CD34+ ECs at two weeks, along with the low vascularisation observed at three days post transplantation, strongly indicates that a large proportion of ECs at the graft site are host derived, as has previously been demonstrated (Vajkoczy et al., 1995, Linn et al., 2003b, Brissova et al., 2004, Nyqvist et al., 2005, Nyqvist et al., 2011, Henriksnas et al., 2012). Notably, both the three day and two week islet grafts analysed in this study consisted of fresh islets, in which a large number of remnant ECs are known to be present (Linn et al., 2003b, Brissova et al., 2004, Nyqvist et al., 2005). Thereby indicating that a large proportion of donor ECs do not survive the immediate post transplantation period, or at least do not remain in the transplanted islet tissue. Indeed, a recent study showed that approximately fifty percent of donor ECs migrate out of the islets into the surrounding tissue stroma (Nyqvist et al., 2011). In support of this, immunostaining of three day islet grafts with CD31 antibodies also shows a similarly low endocrine vascularisation, with a four-fold higher density of CD31+ ECs in the surrounding stroma (see Figure 5.7). It is however acknowledged that the donor ECs would need

to be tagged to confirm their contribution to the revascularisation process in this study. The peripheral distribution of ECs at the graft site has implications for the overall oxygenation of the transplanted islet tissue, as the lack of centrally located ECs is likely to result in a lower oxygen tension towards the centre of the graft, as indicated in a study by Carlsson and colleagues (Carlsson et al., 1998). Importantly, as well as being detrimental to EC survival (Lai et al., 2005), hypoxia can also impair vital aspects of β -cell function, including insulin secretion (Dionne et al., 1993, Vasir et al., 2000) and ultimately lead to β -cell death (Davalli et al., 1996, Biarnes et al., 2002, Miao et al., 2006).

The suboptimal density of CD34+ ECs within the endocrine tissue of two week islet grafts is in agreement with a number of previous studies (Mattsson et al., 2002b, Mattsson et al., 2003, Olsson and Carlsson, 2005), including Mattsson and co-workers who reported that the vascularisation of islets transplanted beneath the kidney capsule was decreased compared to endogenous pancreatic islets, using BS-1 as their EC marker (Mattsson et al., 2002b). Thus, the use of CD34 as an immunohistological marker for investigating ECs in endogenous, isolated/cultured and transplanted islets, results in comparable differences between these groups, to those reported by others utilising alternative markers.

Despite the suboptimal vascular engraftment seen in diabetic recipients of both fresh and cultured islets, the average blood glucose concentrations declined after two weeks in both transplant groups, coinciding with the time point at which islet revascularisation is thought to be completed (Menger et al., 1992, Mendola et al., 1994, Merchant et al., 1997). These observations suggest that there is an improvement in β -cell function as the EC density within the endocrine tissue increases. The enhanced vascularisation of fresh islet grafts is also likely to correlate with the improved glycaemic control, where enhanced vascularisation has been shown to correlate with increased insulin levels in the vascular system (Brissova et al., 2006). The increased EC density observed in the endocrine tissue of islet grafts consisting of fresh islets suggests that the higher vascularisation of freshly isolated as opposed to cultured islets may be of importance. Despite the low vascular density observed in islet grafts consisting of fresh islets three days post transplantation, it has been

suggested that remnant donor ECs are important for attracting host ECs into the transplanted islets (Olsson and Carlsson, 2005) and that they become incorporated into the newly formed microvessels, (Linn et al., 2003b, Brissova et al., 2004, Nyqvist et al., 2005, Nyqvist et al., 2011). Furthermore, it has been suggested that the presence of remnant microvessels in fresh islets may ensure the physical presence of vascular channels, making it easier for host ECs to migrate in to the transplanted islet tissue (Olsson and Carlsson, 2005). Therefore, remnant vascular channels may collapse during culture, leading to the compaction of islets, thereby making it more difficult for host ECs to migrate into the endocrine tissue of cultured islet grafts.

The reasons for inadequate islet revascularisation are likely to be multiple. Notably, during islet development, reciprocal interactions between β -cells and ECs occur, ensuring that they proliferate in accordance with each other (Lammert et al., 2001). Thus, revascularisation of islets which have previously lost their ECs due to the detrimental effects of islet culture and/or transplantation is far from the typical developmental situation. As the vascular engraftment occurs, hypoxia-related angiogenic stimuli are likely to decline. Therefore, after the initial post transplantation period, where angiogenic factors such as VEGF are known to be upregulated in hypoxic tissues (Ferrara et al., 2003), including transplanted islets (Vasir et al., 2001), the balance between pro- and anti-angiogenic factors is no longer likely to favour any further revascularisation. Furthermore, non-specific inflammatory stimuli are decreased with time after the operative procedure and it may well be that the balance between angiogenic and angiostatic factors is simply not favourable for optimal vascular engraftment. Indeed, inhibition of the angiostatic factor thrombospondin-1 was recently shown to enhance islet revascularisation and function (Olerud et al., 2008); evidence that manipulation of this balance can result in better transplantation outcome.

Although fresh islets do appear to produce superior transplantation outcomes to those of cultured islets, in terms of both vascular engraftment and curative capacity, it is clear that improvements are needed in terms of enhancing both the rate and overall extent to which the transplanted islets revascularise. Efforts to reduce chronic ischemic stresses after transplantation should help to prevent the extensive hypoxia-

related β -cell death during the immediate post transplantation period and extend the longer term graft function and survival. Clearly, there is also a need for improved islet culture conditions if a movement towards the use of cultured islets clinically is going to occur, for logistical and safety reasons. Therefore, subsequent experiments in this thesis have focused on enhancing the rate and overall extent to which islets revascularise and have applied a more stringent minimal islet mass model (150 fresh islets instead of 200) in order to correlate any differences in engraftment with graft function. These strategies should aim to make better use of the donor islets that are available, but which are in such short supply clinically.

4 Chapter Four - Mesenchymal stem cells maintain islet organisation and morphology

4.1 Introduction

It was anticipated that islet transplantation would become the treatment of choice for the majority of patients with type 1 diabetes mellitus (T1DM) by 2010 (Serup et al., 2001). Unfortunately, a number of factors have limited the widespread application of islet transplantation, despite the encouraging results of the Edmonton protocol in 2000 (Ichii and Ricordi, 2009, Shapiro et al., 2000) and significant improvements since this. As well as immunological barriers, namely the recurrent autoimmunity and allorejection that prevails, a number of other non-immunological factors contribute to the extensive β -cell death seen during the immediate post transplantation period (Davalli et al., 1996, Biarnes et al., 2002, Miao et al., 2006, Emamaullee et al., 2007). This loss of functional islets during the early post transplantation period limits the success of individual islet grafts and further exacerbates the scarcity of islet tissue.

The transplanted islets are poorly vascularised during the immediate post transplantation period, as shown in Chapter Three and in accordance with previous studies (Menger et al., 1989, Miao et al., 2006). Therefore the islets have to function in a hypoxic microenvironment, which contributes to ischemic cell death and inflammatory events. In addition to these early challenges for transplanted islets, the suboptimal revascularisation also shown in Chapter Three and confirming previous observations (Mattsson et al., 2002b, Mattsson et al., 2003, Olsson and Carlsson, 2005, Lau and Carlsson, 2009), threatens the longer term function and survival of transplanted islets. An improved rate or extent of revascularisation of transplanted islets, or enhancement of islet survival should improve the outcome of islet transplantation.

Mesenchymal stem cells (MSCs) are adult progenitor cells, which can proliferate *in vitro* and give rise to differentiated mesenchymal cell types (Pittenger et al., 1999). MSCs have been isolated from many tissues and may be localised to a perivascular

niche that is present in most, if not all, vascularised organs (Meirelles et al., 2006). MSCs play a major role in tissue repair through localised immunosuppressive effects and through the release of soluble trophic factors to affect neighbouring cells (Xu et al., 2008). These properties make MSCs excellent candidates for improving the survival of transplanted islets. Several recent studies in experimental animals have reported that co-transplantation of islets and MSCs produces superior outcomes to islet-alone grafts (Jacobson et al., 2008, Ding et al., 2009, Solari et al., 2009, Figliuzzi et al., 2009, Park et al., 2009, Longoni et al., 2010, Sordi et al., 2010, Ito et al., 2010, Berman et al., 2010, Li et al., 2010). To date, these beneficial effects have been largely attributed to direct or indirect actions of MSCs in promoting islet survival and function by enhancing graft revascularisation (Figliuzzi et al., 2009, Ito et al., 2010, Sordi et al., 2010) or by suppressing immune or inflammatory responses (Jacobson et al., 2008, Ding et al., 2009, Solari et al., 2009, Longoni et al., 2010, Li et al., 2010).

The kidney subcapsular site is most commonly used for islet transplantation in rodent studies (Merani et al., 2008), although this is known to result in remodelling of the islets within the graft tissue in terms of morphology and composition of endocrine cells (Davalli et al., 1996, Morini et al., 2007, King et al., 2007, Lau et al., 2007), which is associated with cell loss and disruption of normal islet function (Davalli et al., 1996). However, this site is easily accessible in mice and offers the advantage over other sites in that the graft-bearing kidney can be removed by nephrectomy, in order to determine whether cured mice have done so due to the functioning of the graft itself, as opposed to any significant endogenous pancreatic β -cell regeneration and function. In this study we used the renal subcapsular site for implantation of a minimal islet mass. Our aims were to assess in a syngeneic mouse model of diabetes: (1) the effect of co-transplanting kidney-derived MSCs on islet morphology and vascular engraftment; and (2) the function of the transplanted islets.

4.2 Methods

4.2.1 Mesenchymal stem cell isolation and characterisation

I am grateful to Pedro Chagestelles for isolating and characterising the mesenchymal stem cells (MSCs) used in the studies presented in this thesis. Below is a brief description of the methods used.

4.2.1.1 Isolation of kidney-derived mesenchymal stem cells

Kidney-derived MSCs were isolated from male C57Bl/6 mice. Kidneys were rinsed in Ca^{2+} - and Mg^{2+} - free Hanks' balanced salt solution containing 10mmol/l sodium HEPES (Sigma-Aldrich) and cut into small pieces. The fragments were digested with collagenase type I (1mg/ml; Sigma- Aldrich) for 30 to 45 min at 37°C and then triturated with a glass Pasteur pipette. Cells were pelleted by centrifugation for 10 min at 400 x g at room temperature. After this, cells were resuspended in DMEM supplemented with 1% (vol./vol.) penicillin/streptomycin solution (Gibco BRL, Gaithersburg, MD, USA) and 10% (vol./vol.) FCS, seeded in six-well dishes (3ml/well) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 . The medium was changed after 24 hr, with removal of non-adherent cells. When cultures reached confluence, cells were trypsinised and subcultured in new flasks, at passage ratios empirically determined for two subcultures a week. Adipogenic and osteogenic differentiation protocols were then carried out to determine whether the cells had the capacity to differentiate into adipocytes and osteoblasts respectively.

4.2.1.2 Adipogenic and osteogenic differentiation

Adipogenic differentiation was induced by cultivation of confluent cultures in DMEM containing 20% (vol./vol.) FCS, 2.5µg/ml insulin, 100µmol/l indomethacin, 5µmol/l rosiglitazone and 10nmol/l dexamethazone. For osteogenic differentiation, confluent cultures were cultivated in DMEM containing 10% FCS (vol./vol.), 10mmol/l β -glycerophosphate, 5µg/ml ascorbic acid and 10nmol/l dexamethazone. Cultures were maintained in differentiation media for one month with medium changes twice a week. Cell differentiation was analyzed by histological staining with Oil Red O or Alizarin Red S for adipogenic and osteogenic differentiation, respectively, as described previously (Meirelles et al., 2006).

4.2.1.3 Immunophenotyping by flow cytometry

Cells were analysed for the presence of surface markers by flow cytometry. MSCs at the fifth passage were trypsinised, resuspended in PBS and incubated with the following FITC- or phycoerythrinconjugated antibodies: CD11b, CD31, CD44, CD45, CD73, CD90.2 and stem cell antigen-1 (BD Pharmingen, San Diego, CA, USA). After 30 min incubation at 4°C, the cells were washed and resuspended in 0.5ml PBS. Cells were analysed in a FACS calibur cytometer equipped with 488 nm argon laser (BD Pharmingen).

<u>CD</u>	<u>Description</u>	<u>Expression</u>
CD11b	Integrin alpha-M	Monocytes or macrophages
CD31	Platelet EC adhesion molecule (PE-CAM1)	ECs, platelets, neutrophils, monocytes
CD44	Hyaluronan receptor	Lymphocytes
CD45	Leukocyte common antigen (LCA)	Leukocytes
Sca-1	Stem cell antigen-1	Hematopoietic stem cells Activated lymphocytes
CD73	Ecto-5' nucleotidase (NT5E)	Marker of lymphocyte differentiation
CD90.2	THY-1 T-cell antigen	T-cells

Antigens used for the criteria of murine kidney-derived MSCs. Abbreviations: CD, cluster of differentiation number.

4.2.2 Minimal islet mass model

Streptozotocin-diabetic male C56Bl/6 mice were used as recipients. The number of islets transplanted aimed to cure diabetes in only a proportion of diabetic recipients in the control islet-alone transplant group, in order to determine whether MSC co-transplantation would affect this outcome. Based on previous experiments, 150 fresh islets was chosen as a minimal islet mass, because the less stringent use of 200 fresh islets had previously resulted in a 100 percent cure rate by one month post transplantation (see Figure 3.1). Islet + MSC recipients were transplanted with 150

fresh islets and 25×10^4 kidney-derived MSCs of passage 7. Animals were considered cured if their non-fasting glucose concentrations were lower than 11.1mmol/l for two consecutive readings without reverting to hyperglycaemia on any subsequent date.

4.2.3 Evaluation of graft morphology

For each animal five to nine tissue sections from different graft areas were evaluated for the total endocrine area in each individual graft section. To evaluate the extent of islet fusion the number of individual endocrine aggregates in each graft section was measured. An individual endocrine aggregate was defined as an area of insulin-positive tissue separated from any other adjacent insulin-positive tissue by $\geq 50\mu\text{m}$ of non-endocrine tissue (insulin-negative). Total endocrine area refers to the sum of the area of all endocrine aggregates within an individual graft section. The demarcation of the islet graft was taken as the area of endocrine and non-endocrine tissue between the renal parenchyma and the kidney capsule. Area and diameter were determined using Image J software (<http://rsbweb.nih.gov/ij/>).

4.2.4 Evaluation of vascular density

The endothelial cell (EC) density of transplanted islets was quantified as described in section 3.2.4. The morphology of the islet + MSC grafts was clearly different to that of islet-alone grafts, with a greater proportion of the graft occupied by non-endocrine tissue. For the studies presented in this chapter and all subsequent experiments presented in this thesis, the EC density of the non-endocrine component (within the field of view from images taken at a magnification of x400) of the graft was determined, in addition to the endocrine component of the graft. For each animal \geq five tissue sections from different graft areas were evaluated.

4.2.5 Van Geison staining

Islet graft sections were stained with van Geison (Sigma) in order to produce differential staining between cytoplasmic proteins (staining yellow) and collagen fibres (staining pink). Tissue sections were de-waxed and re-hydrated as detailed in section 2.9.5. Tissue sections were incubated in Van Geison stain for 20 min. The Van Geison stain was flicked off and a cotton bud used to soak up any excess stain, before

allowing sections to dry for approximately 2 mins without washing in water (to prevent the stain being washed out). Sections were then dehydrated and mounted as detailed in 2.9.5.

4.2.6 Hormone measurement of islet grafts

The islet graft-bearing kidneys or pancreas were homogenised in 2ml acid ethanol (0.18 mol/l in 70% [vol./vol.] ethanol) then sonicated on ice, for 3x 5 second pulses, output 10-14 and hormones extracted overnight at 4°C. Samples were then stored at -20°C until a later time point. Each sample was diluted with borate buffer to an appropriate concentration for the detection limits of the radioimmunoassay (RIA). Tissue insulin, glucagon and somatostatin contents were measured by RIA (as detailed in section 2.10.4) and a commercially available somatostatin RIA kit (Euro-Diagnostica, Malmö, Sweden).

4.3 Results

4.3.1 Mesenchymal stem cell characterisation

Kidney-derived MSCs were characterised by Pedro Chagestelles (Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil). The characteristics of the adherent cells isolated from the whole kidney of male C57Bl/6 mice and expanded *in vitro* were similar to those previously described (Meirelles et al., 2006).

4.3.2 Morphology and immunophenotype of kidney-derived MSCs

The cells presented morphological characteristics of MSCs, with a clear fibroblastic spindle-shaped morphology evident when visualised by phase contrast microscopy (Figure 4.1a). Their identity was confirmed by flow cytometry immunophenotyping. Thus, the cells were negative for CD11b, CD31 and CD45, which are markers of macrophages, ECs and hematopoietic cells, respectively, but positive for CD44 and stem cell antigen-1 (Figure 4.1b), which are characteristic of MSCs and the minimum immunophenotypic characterisation required for murine MSCs. Additionally, the cells were positive for CD73 and CD90.2, which are also characteristic of murine MSCs (Figure 4.1c).

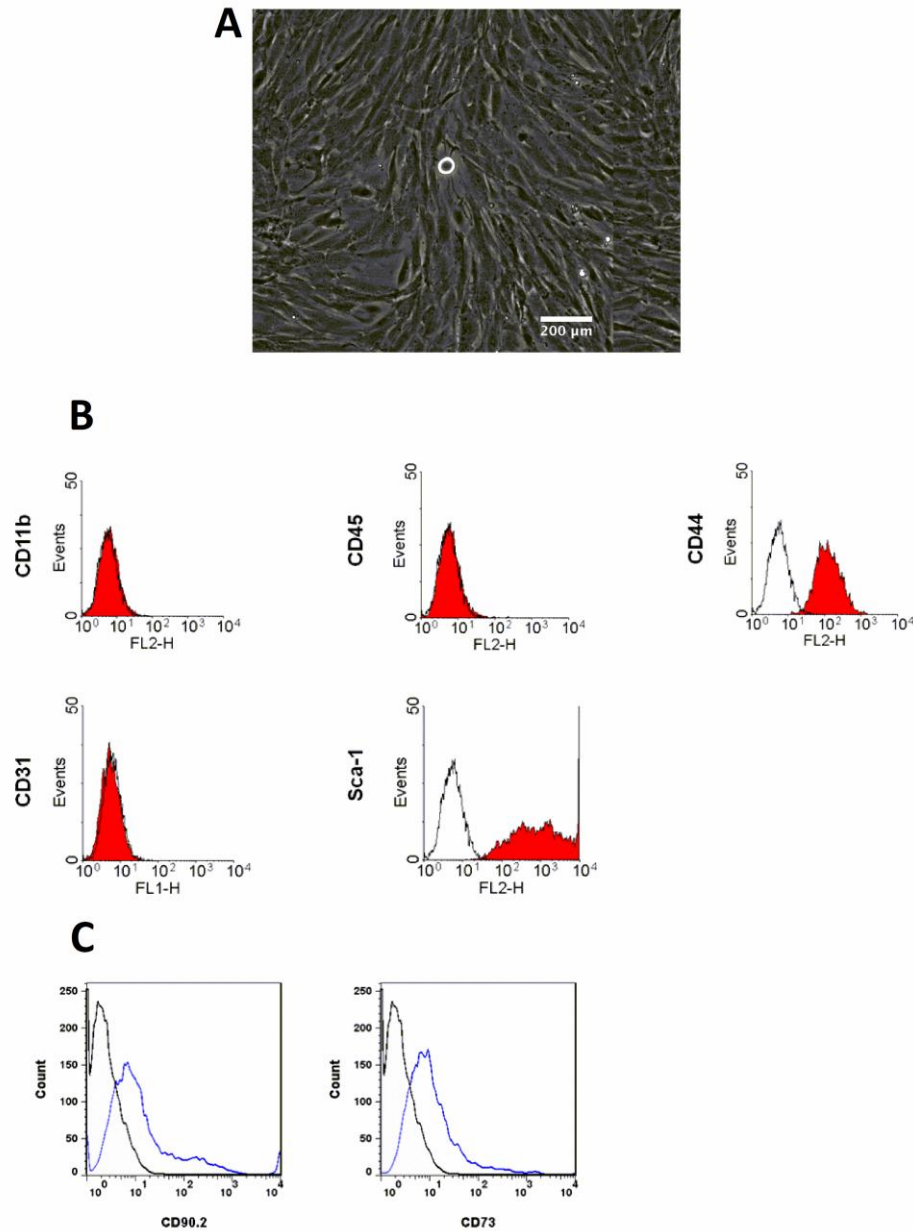


Figure 4.1 Morphology and immunophenotype of mouse kidney-derived MSCs. **A.** Micrograph of P7 kidney-derived MSCs, showing the fibroblast-like spindle shaped morphology typical of MSCs. **B.** Immunophenotypic profiles of MSCs derived from C57Bl/6 mouse kidney tissue at passage 5. Flow cytometry histograms showing the expression (shaded red) of selected molecules essential for the characterisation of murine MSCs (CD11b, CD45, CD44, CD31, Sca-1) compared with negative isotype controls (unshaded peaks). **C.** Flow cytometry histograms showing the expression (blue line) of additional molecules confirming the MSC phenotype of the cells (CD90.2 and CD73), compared with negative isotype controls (black line), as in B.

4.3.3 Adipogenic and osteogenic differentiation of kidney-derived MSCs *in vitro*

Intracellular lipid droplets were evident in the cells that had been cultured in adipogenic differentiation medium for one month, as was evident through histological staining with Oil Red O (Figure 4.2a). For cells cultured in osteogenic medium, mineralised nodules in which calcium accumulates were shown to be present, as shown by histological staining with Alizarin Red S (Figure 4.2b). As a control to determine whether cells were able to spontaneously differentiate into adipocytes or osteoblasts, cells from the same vial as those that were cultured in adipogenic or osteogenic medium, were cultured in DMEM supplemented with 10% FCS (vol./vol.) in the absence of any additional adipogenic or osteogenic supplements. Control cells did not spontaneously differentiate into adipocyte- (Figure 4.2c) or osteoblast-like cells, as shown in Figure 4.2d.

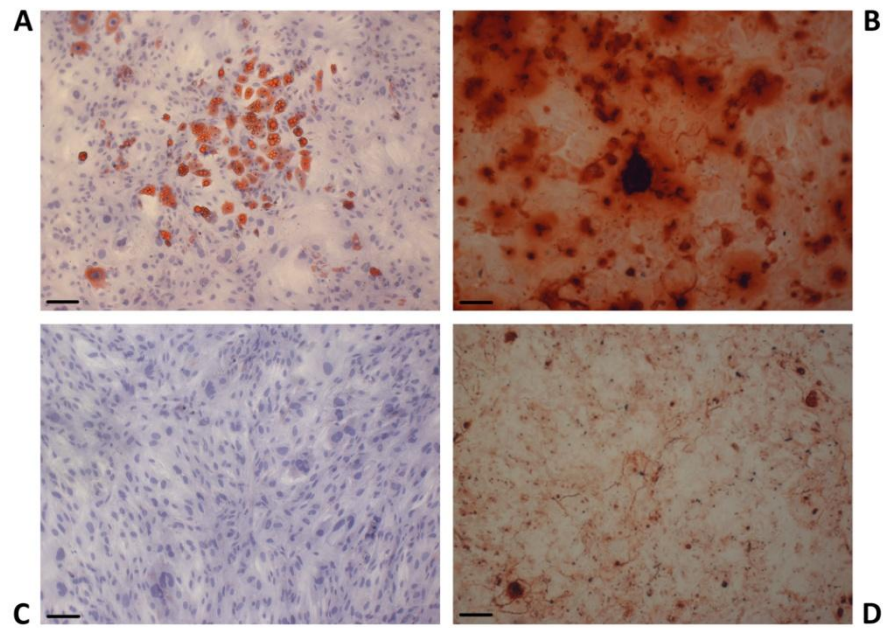


Figure 4.2 Adipogenic and osteogenic differentiation of mouse kidney-derived MSCs. Kidney-derived MSCs at passage 8 submitted to adipogenic (a) and osteogenic (b) protocols and respective controls (c, d) after staining with Oil Red O (a, c) and Alizarin Red S (b, d), original magnification x100, scale bars are 20µm.

4.3.4 Graft function over a one month monitoring period

Co-transplantation of islets with kidney-derived MSCs produced superior transplantation outcomes to islet-alone grafts, as shown in Figure 4.3. The average blood glucose concentrations of mice transplanted with islets + MSCs were significantly lower than in mice transplanted with islets alone at 3, 7, 14, 21 and 28

days after transplantation (Figure 4.3a). After one month, only 8 percent of mice transplanted with islets + MSCs had not reverted to hyperglycaemia, compared with 58 percent of mice transplanted with islets alone (Figure 4.3b). The average time to reverse hyperglycaemia for islet + MSC grafts was 7 ± 2 days, with islet alone recipients taking significantly longer (17 ± 2 days, $p < 0.001$, $n=13$). The median time to attain normoglycaemia was 4 and 17 days for islet + MSC and islet-alone grafts, respectively. There were no significant differences in the weights of mice in either transplant group on day 0 (24.2 ± 0.7 and 23.2 ± 0.3 g for islet-alone and islet + MSC recipients, respectively, $n=13$, $p > 0.2$) or at one month after transplantation (25.9 ± 0.7 and 26.1 ± 0.4 g, $n=13$, $p > 0.2$). MSC-alone grafts did not lower blood glucose concentrations in streptozotocin-induced diabetic mice (day 0, 23.7 ± 1.6 mmol/l; day 3, 23.4 ± 4.1 mmol/l, $n=3$), which had to be killed after a three day monitoring period due to excessive weight loss. Thus, MSCs alone had no capacity to reverse streptozotocin-induced diabetes. At one month after transplantation, non-fasted serum insulin concentrations were 432 ± 114 pmol/l in islet + MSC mice vs 300 ± 47 pmol/l in islet-alone mice ($n=11$, $p = 0.25$, Student's t test). This was associated with lower blood glucose in the islet + MSC mice than in the islet-alone mice (8.2 ± 0.8 mmol/l vs 17.1 ± 3.2 mmol/l, $n=11$, $p = 0.01$, Student's t test). At this time-point, IPGTTs were carried out in all transplanted mice with blood glucose <11.1 mmol/l and in weight-matched non-diabetic, non-transplanted controls. Glucose tolerance was similar in both transplant groups, but impaired in comparison to non-transplanted controls (Figure 4.3c). At two days after the IPGTT, some of the mice with blood glucose <11.1 mmol/l in each transplant group were nephrectomised, with all nephrectomised mice reverting to hyperglycaemia within two days (Figure 4.3d).

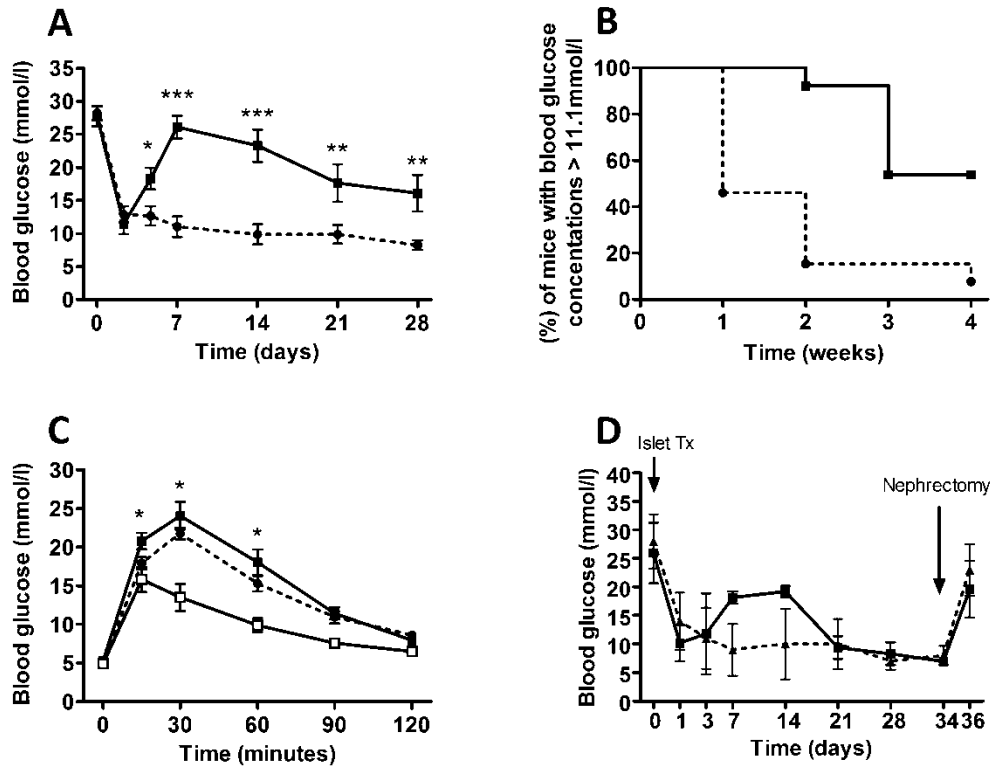


Figure 4.3 Graft function. **A.** Blood glucose concentrations of mice transplanted with 150 islets alone (continuous line) or those co-transplanted with 150 islets + 25×10^4 kidney-derived MSCs (dashed line); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs mice transplanted with islets + MSCs (RM ANOVA with Bonferroni post hoc test, $n=13$). **B.** Percentage of mice remaining diabetic (blood glucose concentration > 11.1 mmol/l) after transplantation as above (**a**); $p = 0.0005$ Kaplan Meier, $n=13$ for both transplant groups. **C.** IPGTTs in all mice with reversed hyperglycaemia after transplantation as above; IPGTT was conducted four weeks after transplantation, * $p < 0.05$ vs non-diabetic non-transplanted controls (RM ANOVA with Bonferroni post hoc test, $n=6-12$), black squares, islets alone; black circles, islets + MSCs; white squares, control weight-matched, non-diabetic non-transplanted mice. **D.** Blood glucose concentrations of cured mice only after islet transplantation (day 0) and after removal of the graft bearing kidney at 28 days post transplantation ($n=2-5$). **A, C** Data expressed as means \pm SEM, **D.** Data expressed as means \pm SD.

4.3.5 Pancreatic islet morphology at one month post transplantation

The STZ-pancreas of mice from both transplant groups at one month post transplantation was sectioned and the endocrine composition assessed in comparison to the pancreatic islets of control age-matched male C57Bl/6 mice treated with citrate buffer (the buffer in which STZ is dissolved). Immunostaining of β -cells with insulin antibodies showed that the majority of the islet area was insulin-positive in control citrate-treated mice after one month (Figure 4.4a), with very few endogenous β -cells

remaining in islet-alone (Figure 4.4b) and islet + MSC (Figure 4.4c) transplanted mice. The typical rim of glucagon-positive α -cells was present in islets of citrate-treated mice (Figure 4.4d), whilst α -cells made up the majority of the islet area for islet-alone (Figure 4.4e) and islet + MSC recipients (Figure 4.4f). Quantification of endocrine cells showed that the percentage of β -cells was approximately 80 percent in citrate-treated mice, as expected, with only 20 percent of the islet cell mass consisting of β -cells for mice in both transplant groups (Figure 4.4g). Conversely, the percentage of α -cells was approximately 20 percent in citrate-treated mice and 60 percent for transplanted mice (Figure 4.4h), confirming the selectivity of streptozotocin for β -cells.

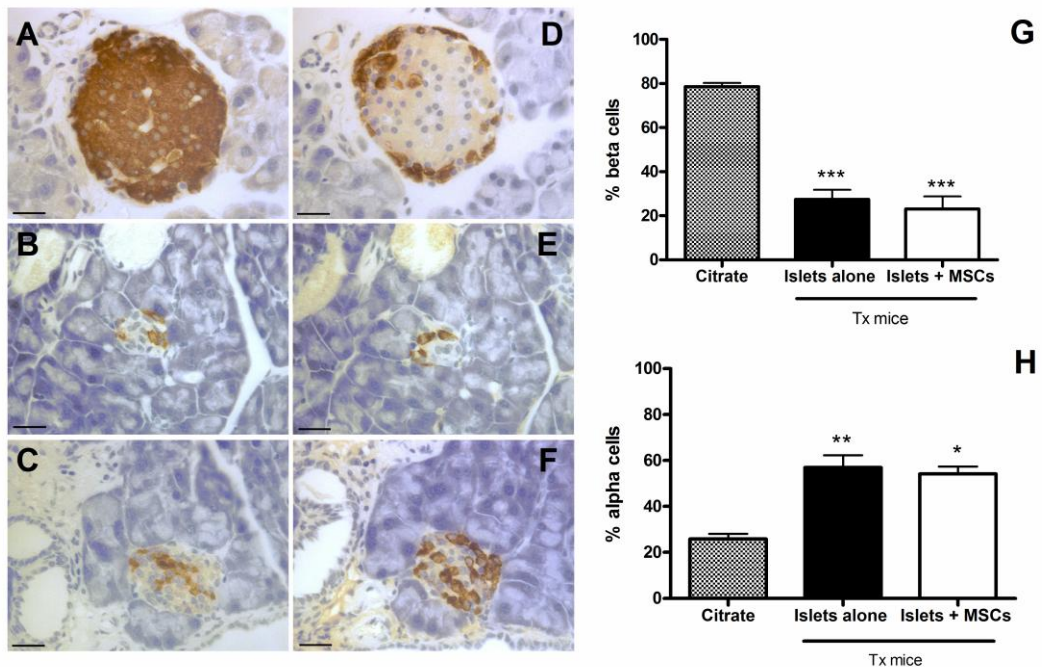


Figure 4.4 Endogenous β -cell regeneration. Immunostaining of pancreas sections in control citrate-treated mice after one month (**a, d**), mice transplanted with islets alone (**b, e**) and mice transplanted with islets + MSCs (**c, f**) at one month, with insulin (**left hand panel**) and glucagon antibodies (**middle panel**), original magnification x400, scale bars are 25 μ m. **G, H** Endocrine composition of pancreas in STZ- induced diabetic mice transplanted with islets alone (black bars) or islets + MSCs (white bars) and control non-diabetic, non-transplanted citrate-treated mice (white and black hashed bars) **G, H** Percentage insulin positive β -cells and percentage glucagon positive α -cells in the same islets; * p < 0.05, ** p < 0.01, *** p < 0.001 vs citrate-treated animals (one-way ANOVA with Bonferroni post hoc test, n=5-9 animals per group).

4.3.6 Graft morphology at one month post transplantation

Figure 4.5 shows the typical morphology of graft material retrieved one month after transplantation, demonstrating that islets co-transplanted with MSCs maintained a morphology that more closely resembles that of endogenous islets in the pancreas, in contrast to the amorphous mass of endocrine tissue that forms in islet-alone grafts. Insulin immunostaining of islet-alone grafts revealed a single amorphous endocrine mass in the majority of sections analysed (Figure 4.5a, c) where the transplanted islets have fused to form aggregated islet tissue. In contrast, there were rarely signs of any fusion between individual islets in the grafts of islet + MSC recipients. In these grafts, insulin immunostaining revealed endocrine aggregates with the appearance of normal islets separated by extensive areas of non-endocrine tissue (Figure 4.5b, d). Although the total area of endocrine tissue per section (immunostained for insulin) was similar in islet-alone and islet + MSC grafts (Figure 4.5e), it was clear that the graft morphology was different. We quantified the extent of islet fusion within the grafts of both transplant groups by measuring the average area of each individual endocrine aggregate, defined as an individual mass of islet tissue that was separated from any adjacent endocrine aggregate by $\geq 50\mu\text{m}$ in each graft section. The average area of each single endocrine aggregate in islet + MSC recipients was approximately fourfold smaller than that of the aggregates in islet-alone recipients (Figure 4.5f). The total graft area (endocrine + non-endocrine tissue) was higher in islet + MSC grafts than in islet-alone grafts ($322,596 \pm 38,919$ vs $134,546 \pm 14,941\mu\text{m}^2$, $p < 0.001$, Student's t test, $n=4-6$). Considering that the area of endocrine tissue was similar in both grafts, it was clear that the islet + MSC grafts contained large areas of non-endocrine tissue.

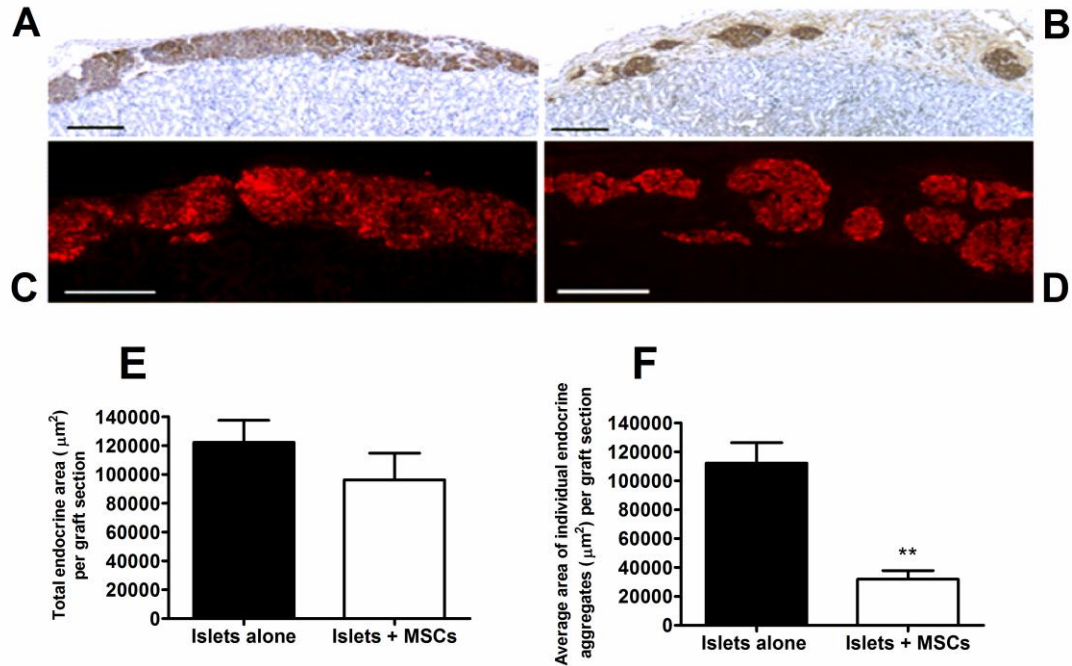


Figure 4.5 Graft morphology. **A, C** Representative sections of islet-alone grafts and **(b, d)** islet + MSC grafts at one month post transplantation. **A.** Islet-alone graft, where islets have aggregated to form a single amorphous endocrine mass. **B.** Islet + MSC graft, where individual endocrine aggregates are separated by extensive areas of non-endocrine tissue, original magnification **(a, b)** x40, scale bars are 100 μm . **C.** At a higher magnification (x100), islet-alone graft shows that the rounded morphology of individual islets can no longer be discerned, in comparison with **(d)** islet + MSC graft, where even when islets have aggregated, they still maintain a morphology comparable to that of endogenous islets in the pancreas, with individual islets still clearly distinguishable from each other, scale bars are 100 μm **(c, d)**. **E.** Total endocrine area in graft sections; n=4–6 animals per group, $p > 0.2$, Student's t test. **F.** Average individual endocrine aggregate area in graft sections; n=4–6 animals per group, $**p < 0.01$ vs islet-alone grafts, Student's t test.

Alpha smooth muscle actin (α -SMA) is a marker which has previously been shown to stain murine MSCs (da Silva et al., 2006). Therefore, graft sections from islet + MSC recipients were immunostained with α -SMA antibodies as an indicator of the presence of MSCs at one month. Analysis of the non-endocrine tissue surrounding the islets in MSC co-transplanted recipients at one month post transplantation showed very few α -SMA-positive cells, as shown in Figure 4.6. Dual immunostaining with insulin and α -SMA antibodies showed the small endocrine aggregates with occasional areas of positive staining for α -SMA in the non-endocrine component of the graft (Figure 4.6a). Perivascular cells within the kidney tissue itself also stained positively with α -SMA as expected. The non-endocrine component of islet-alone grafts, making up

only 9.2 percent of the total graft area, appeared to consist of very tightly packed collagen fibres as indicated by van Geison stain (Figure 4.6b). In islet + MSC grafts, the non-endocrine component made up 63.4 percent of the total graft tissue and was largely extracellular tissue, consisting of more loosely packed fibres, staining positively with van Geison, which strongly indicates the presence of collagen (Figure 4.6c).

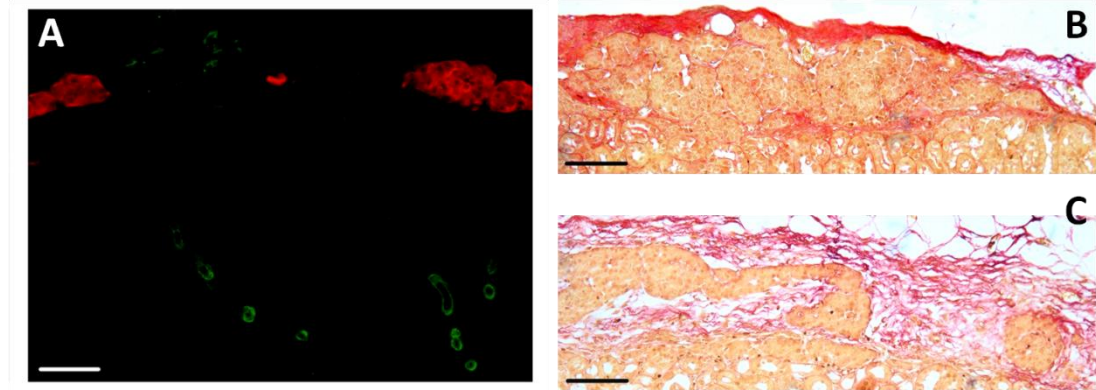


Figure 4.6 Graft non-endocrine component composition. **A.** Representative sections of islet + MSC grafts at one month post transplantation, showing insulin-positive (red) endocrine aggregates, with very few α -SMA-positive (green) cells present in the non-endocrine component of the graft, original magnification x40, scale bar is 100 μ m. **B.** Islet-alone graft at one month stained with van Geison, indicating the presence of a small number of tightly packed collagen fibres at the graft periphery. **C.** Large areas of non-endocrine tissue present in islet + MSC grafts consisting of loosely packed collagen fibres, as indicated by van Geison stain, original magnification x100, scale bars are 100 μ m, (b, c).

4.3.7 Graft composition at one month post transplantation

Immunostaining for glucagon-positive α -cells indicated that the normal core-mantle segregation of islet endocrine cells was altered in the grafts of islet-alone recipients, as shown in Figure 4.7a. In contrast, the majority of α -cells in islet + MSC grafts were located at the periphery of individual islets (Figure 4.7b). In both transplant groups, somatostatin-positive δ -cells were dispersed among the α -cells. Therefore the δ -cells were distributed throughout the islet-alone grafts (Figure 4.7c), whereas they were primarily located in the islet mantle in the islet + MSC grafts (Figure 4.7d). Analysis of the endocrine composition of the grafts at one month post transplantation showed a significantly higher percentage of α -cells in non-cured islet-alone recipients, compared to cured islet + MSC recipients (Figure 4.7e), whilst the percentage of δ -cells was comparable between transplant groups (Figure 4.7f)

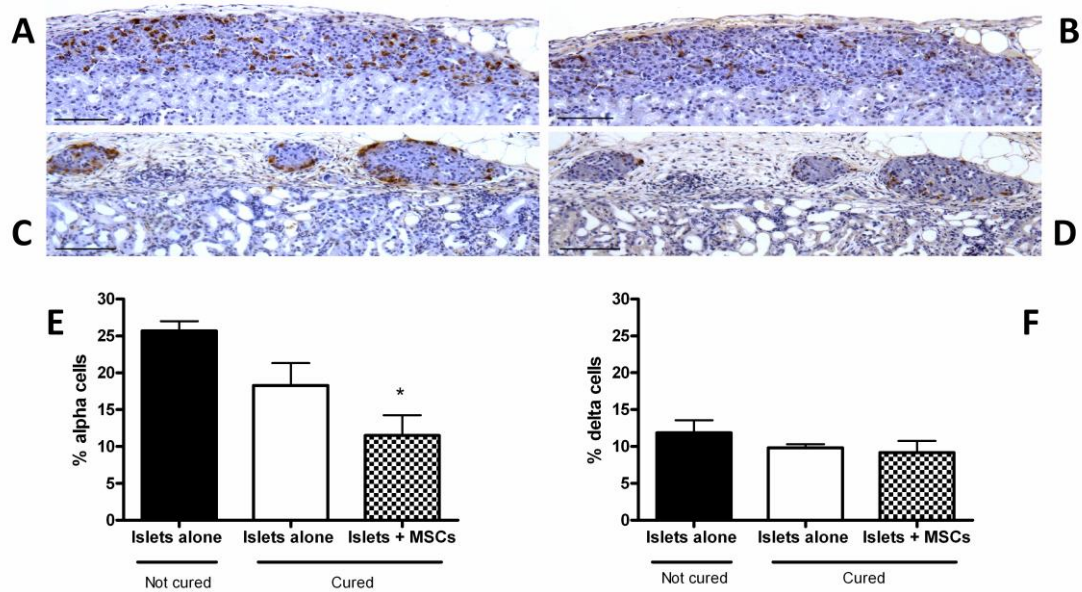


Figure 4.7 Graft composition. Distribution of glucagon-positive α -cells and somatostatin-positive δ -cells. At one month after transplantation, consecutive sections were stained with glucagon (a, b) or somatostatin (c, d) antibodies, in grafts consisting of islets alone (a, c) or islets + MSCs (b, d). Images are representative of sections from four to six different animals, original magnification $\times 100$, scale bars are $100\mu\text{m}$. E. The percentage of α -cells and (f) δ -cells in the same graft sections were quantified, $n=4-6$, $*p < 0.05$ vs non-cured islet-alone recipients, one-way ANOVA with Bonferroni post hoc test.

4.3.8 Hormone content of grafts and pancreata

At one month after transplantation there was considerable variation in the graft insulin content between animals, ranging from 3.0 to 15.2 and 7.7 to 21.1 $\mu\text{g}/\text{graft}$ for islet-alone and islet + MSC recipients respectively. The mean insulin content of islet + MSC grafts was approximately 60 percent higher than in islet-alone grafts, but this was not statistically significant ($p > 0.05$; Figure 4.8a). The graft glucagon and somatostatin contents were similar between transplant groups (Figure 4.8b, c). The insulin content of the pancreas in all STZ-treated mice was around ten times lower than that of the grafts and approximately one percent that of native pancreatic islets (1.4 ± 0.3 and $142.6 \pm 24.5\mu\text{g}/\text{pancreas}$, STZ pancreata at one month post transplantation vs age matched control non-diabetic non-transplanted mice pancreata, $n= 4-5$, $p = < 0.0001$, Student's t test), with no differences in the STZ-pancreas insulin content for mice transplanted with islets alone or islets + MSCs (Figure 4.8d). The STZ-pancreas glucagon content was approximately two-fold higher than that of

the grafts for both transplant groups (Figure 4.8e) and the somatostatin content approximately three-fold higher (Figure 4.8f), with no differences between transplant groups observed, indicating the selectivity of STZ for β -cells as shown with the immunostaining shown in Figure 4.4.

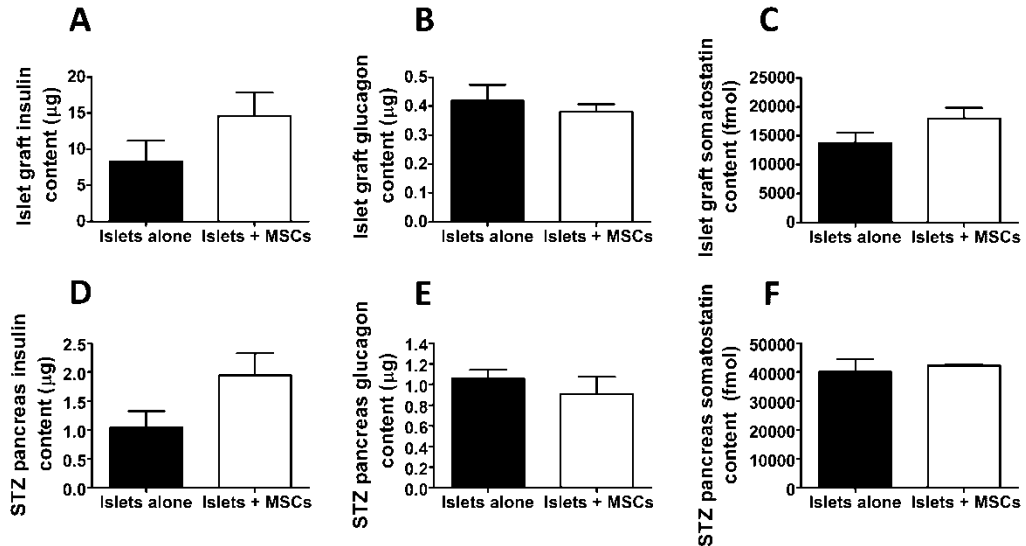


Figure 4.8 Hormone content of grafts and pancreata. **A.** Insulin, **(b)** glucagon and **(c)** somatostatin content of grafts at one month after transplantation. Mice were transplanted with 150 islets alone (black bars) or 150 islets + 25×10^4 kidney-derived MSCs (white bars). **D.** Insulin, **(e)** glucagon and **(f)** somatostatin content of STZ-pancreata in the same transplanted mice at one month post transplantation, $n=4$, $p > 0.05$, Student's t test.

4.3.9 Vascular density at one month post transplantation

CD34 antibodies were used to immunostain microvascular endothelium in endogenous pancreatic islets (Figure 4.9a) and in islet grafts beneath the kidney capsule (Figure 4.9b-d). The distribution of CD34+ ECs in islet + MSC grafts was similar to that of islets in the pancreas, with ECs located throughout the islet mass (Figure 4.9b). In contrast, islet-alone grafts contained areas of endocrine tissue that were devoid of any ECs, with no detectable differences being observed between hyperglycaemic and normoglycaemic recipients (Figure 4.9c, d). We quantified the number of ECs in the endocrine and non-endocrine tissue in the field of view by counting CD34+ cells. The vascular density of the endocrine tissue in grafts consisting of islets + MSCs was significantly higher than that of islet-alone grafts, as shown in Figure 4.9e. However, the non-endocrine tissue in islet-alone grafts had a

markedly higher vascular density than the non-endocrine tissue of islet + MSC grafts (Figure 4.9e).

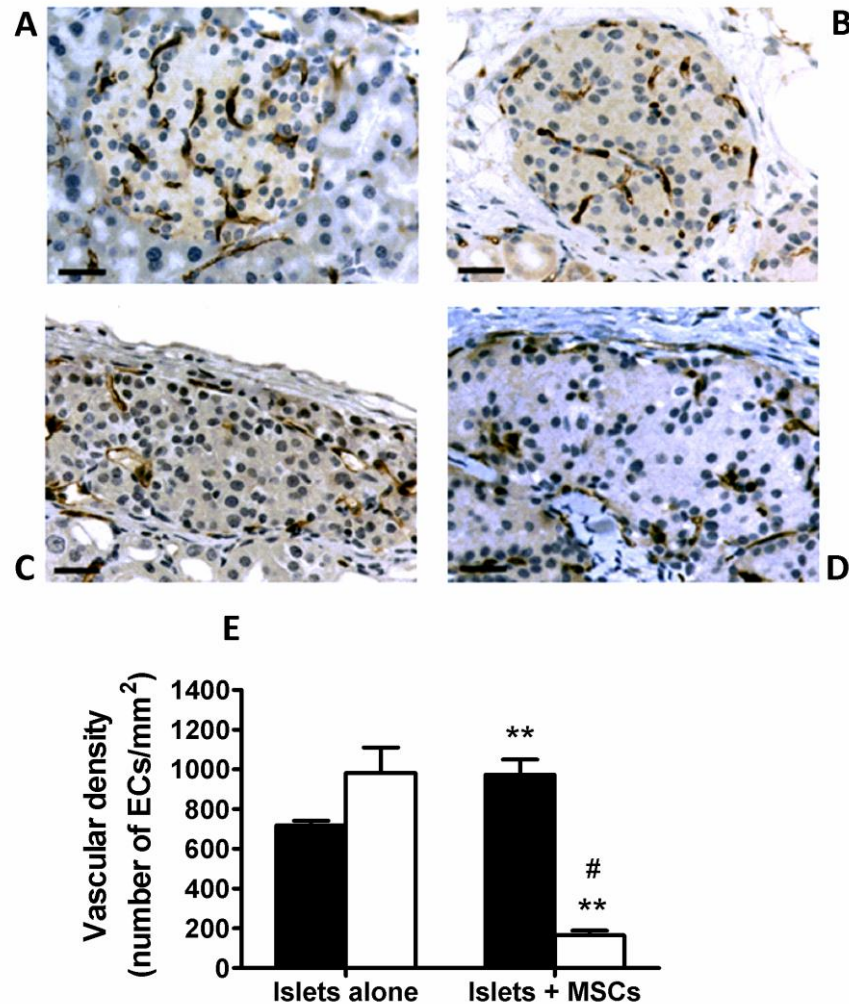


Figure 4.9 Vascular density Immunostaining of ECs with CD34 antibodies in endogenous pancreatic islets (a) and in grafts of islet + MSC recipients with blood glucose <11.1 mmol/l at one month after transplantation (b), of islet-alone hyperglycaemic recipients (c) and of islet-alone recipients with blood glucose <11.1 mmol/l (d), original magnification x400, scale bars are 25µm. **E.** Vascular density of endocrine components (black bars) and non-endocrine components (white bars) in one month grafts consisting of islets alone or islets + 25×10^4 kidney-derived MSCs, $^{**}p < 0.01$ vs islet-alone and $^{\#}p < 0.01$ vs endocrine tissue within the same transplant group, by two-way ANOVA with Bonferroni post hoc test.

Consecutive grafts sections were immunostained with CD34 and CD31 antibodies, as shown in Figure 4.10. There were areas of endocrine tissue within islet-alone grafts that were devoid of CD34+ ECs (Figure 4.10a), with very few areas of endocrine tissue lacking CD34+ ECs in the grafts of islet + MSC recipients (Figure 4.10b), as

also shown in the previous figure. The distribution of CD31⁺ ECs within the endocrine tissue of both islet-alone (Figure 4.10c) and islet + MSC recipients (Figure 4.10d) was comparable to that seen using CD34 as a microvascular EC marker. Quantification of cells staining positively for CD34 and CD31, showed that the number of both CD34⁺ and CD31⁺ ECs was elevated within the endocrine tissue of islet + MSC grafts, compared to that of islet-alone recipients. In accordance with the results obtained using CD34 as an EC marker, there was also a significantly higher number of CD31⁺ ECs in the non-endocrine tissue of islet-alone grafts, compared to that of islet + MSC grafts. There was also a subset of CD31⁺ ECs observed within the non-endocrine tissue surrounding the islets that did not stain positively for CD34 in the grafts of islet + MSC recipients (Figure 4.10e).

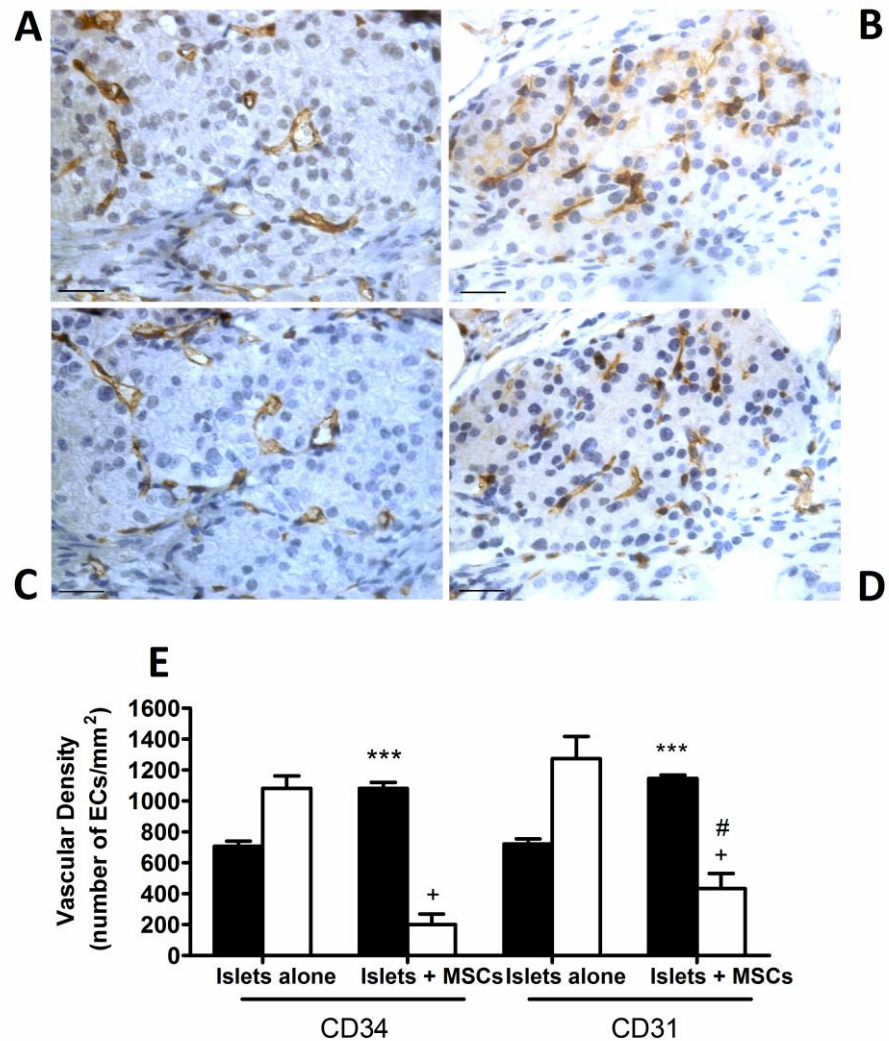


Figure 4.10 Consecutive one month graft sections immunostained with CD34 and CD31 antibodies Consecutive graft sections consisting of islets-alone (a, c) or islets + MSCs (b, d), were stained with CD34 (a, b) or CD31 (c, d) antibodies and are representative of sections from 4-6 different animals in each transplant group, original magnification x400, scale bars are 25µm. **E.** Vascular density of endocrine components (black bars) and non-endocrine components (white bars) of one month grafts consisting of islets alone or islets + MSCs, ⁺⁺ $p < 0.05$ vs endocrine tissue within the same marker and transplant group, ^{**} $p < 0.01$ vs islet-alone grafts within the same tissue type and marker, [#] $p < 0.01$ vs CD34 immunostained sections in same tissue type and transplant group, $n=4$, two-way Anova with Bonferroni post hoc test.

4.4 Discussion

MSCs have a number of functional properties that make them ideal cellular candidates for improving islet transplantation outcome. Their regenerative properties (Uccelli et al., 2008) imply that they may help to enhance β -cell regeneration in the endogenous pancreas and/or aid the engraftment of transplanted islets, thus potentially increasing β -cell survival and function. A syngeneic minimal islet mass model was used to demonstrate that co-grafting kidney-derived MSCs with islets increased the rate and number of recipients attaining normoglycaemia by one month after transplantation. Several reports have indicated that MSCs have beneficial effects in different transplantation models, including islet grafts (Jacobson et al., 2008, Figliuzzi et al., 2009, Ding et al., 2009, Solari et al., 2009, Longoni et al., 2010, Ito et al., 2010, Berman et al., 2010, Sordi et al., 2010, Li et al., 2010, Kim et al., 2011) and several different mechanisms have been thought to account for these effects, some of which are unlikely to explain the results presented in this study.

It is debatable whether MSCs are therapeutically useful for the treatment of T1DM, because of their ability to cause β -cell regeneration in the endogenous pancreas. Recent studies in which MSCs were administered either alone or in a co-transplant setting in rodents, provide evidence both for (Lee et al., 2006, Dong et al., 2008, Ezquer et al., 2008) and against (Urban et al., 2008, Berman et al., 2010) this as a potential mechanism for the observed beneficial effect of MSCs on glycaemic control in this study. Four separate methods were utilised to investigate the possibility that MSCs may have improved glycaemia due to increased regeneration of endogenous pancreatic β -cells: 1) Cured mice were nephrectomised to determine whether glycaemic control was maintained in the absence of the graft bearing kidney; 2) The insulin content of the STZ-pancreata was assessed as an estimation of β -cell number in the pancreas; 3) Immunostaining of STZ-pancreas sections with both insulin and glucagon antibodies was carried out to determine the composition of the pancreatic islets in STZ-diabetic recipients and provide additional information regarding β -cell number and; 4) Mice were transplanted with MSCs alone to determine whether there was any capacity for MSCs to reduce blood glucose concentration in STZ-diabetic mice in the absence of transplanted islets. Together, these experiments conclusively showed that endogenous β -cell regeneration is not the mechanism by which MSCs

were improving glycaemic control in this study, with mice consistently reverting to hyperglycaemia post-nephrectomy and MSC co-transplantation exerting no significant effect on the very low levels of pancreatic insulin and β -cell numbers one month after induction of hyperglycaemia. The presence of glucagon-positive α -cells in the STZ-pancreata of both transplant groups strongly indicates that STZ is selectively killing pancreatic β -cells as expected. Moreover, the presence of α -cells is important, as it shows that the low levels of insulin positivity within the same islet sections are not due to severe β -cell degranulation, which could potentially lead to false negative results for the detection of any remaining β -cells. The hormone content data provides further confirmation regarding the selectivity of STZ and shows that MSCs do not exert any influence on the number or composition of endocrine cells in the pancreas itself. MSCs transplanted alone were unable to lower blood glucose concentrations in STZ-treated mice, indicating that the MSCs were exerting beneficial effects on the transplanted islets, essentially acting as 'islet helper cells'.

Other studies have suggested that the immunosuppressive properties of MSCs may enhance islet survival after transplantation by secreting cytokines (Solari et al., 2009, Longoni et al., 2010, Berman et al., 2010, Kim et al., 2011) or metalloproteases (Ding et al., 2009). This mechanism may be important in allogeneic grafts (Jacobson et al., 2008, Ding et al., 2009, Solari et al., 2009, Longoni et al., 2010, Berman et al., 2010, Li et al., 2010, Kim et al., 2011), but it is unlikely that immunomodulation is the sole mechanism accounting for the beneficial effects of MSCs seen throughout the one month monitoring period, in our syngeneic transplantation model. It is however possible that MSCs may help to dampen down non-specific inflammatory processes in the early post transplantation period (Ryan et al., 2007, Krampera et al., 2006), where levels of pro-inflammatory cytokines, such as IFN- γ , IL-1 β and TNF- α are known to be elevated (Nagata et al., 1990, Barshes et al., 2005, Montolio et al., 2007).

MSCs secrete several trophic factors, many of which could have positive effects on islet cell viability and function in a co-transplant model (Figliuzzi et al., 2009, Park et al., 2009, Ito et al., 2010). For example, *in vitro* co-culture experiments have shown that MSCs increase islet viability by upregulating anti-apoptotic genes and increase insulin secretory function by modulating ATP content (Park et al., 2009). In

accordance with these studies, our kidney MSCs were also able to potentiate glucose induced insulin secretion *in vitro* when islets were cultured together with MSCs using a direct contact co-culture system (see Figure 7.5). Notably, α -SMA immunostaining of islet-MSC graft sections at three days post transplantation indicated the presence of MSCs in the immediate vicinity of the islets at this time point, suggesting that the beneficial paracrine influences of MSCs are likely to be particularly important in the early post transplantation period (detailed in Chapter Five), in contrast to the likelihood of this at one month where there was little evidence of remaining MSCs at the graft site. This is of particular importance as improved glycaemic control in the initial days after transplantation is known to correlate with an enhanced rate of islet revascularisation (Vasir et al., 2000) and better long term transplantation outcome (Warnock and Rajotte, 1988, Kaufman et al., 1990, Keymeulen et al., 1992, Tobin et al., 1993, Merino et al., 1997, Raurell et al., 1999, Emamaullee and Shapiro, 2006).

It is notable that there is a greater percentage of α -cells in the grafts of non-cured islet-alone recipients, compared to cured islet + MSC recipients at one month. Taken together with the similar percentage observed between transplant groups at three days post transplantation (see Figure 5.6), this may indicate that there was greater β -cell death in non-cured islet-alone recipients between 3 days and one month post transplantation. In accordance, Biarnés and colleagues showed that β -cell apoptosis was higher in mice that remained hyperglycaemic at thirty days post transplantation, compared with normoglycaemic mice, who had low levels of β -cell apoptosis; similar to control pancreatic islets (Biarnes et al., 2002). Similarly, Davalli and colleagues also reported a selective decrease in β -cell mass of human islets transplanted into diabetic nude mice, with the most severe loss of β -cells occurring in the first fifteen days post transplantation, but continued β -cell loss between fifteen and thirty days after the revascularisation process is completed (Davalli et al., 1995). The paracrine effects of MSCs alone are unlikely to account for the beneficial effects of MSCs observed in this study. Thus, the presence of co-transplanted MSCs did not cause significant increases in graft insulin content and serum insulin at one after transplantation, suggesting that the enhancing effects of MSCs on graft function may involve other factors.

MSC co-transplantation enhanced the ability of islet grafts to reverse hyperglycaemia, but did not improve glucose tolerance after reversal of hyperglycaemia, in contrast to a recent report where MSC co-transplantation was shown to improve glucose tolerance as well as the curative capacity of islet grafts (Ito et al., 2010). Islet-alone and islet + MSC grafts both showed similar degrees of impaired glucose tolerance when compared with non-transplanted control animals. This is presumably, at least in part, a consequence of the deliberately low insulin content in the minimal mass islet grafts. Additionally, it has been suggested that the reduced vascular density in transplanted islets may result in a lower flux of glucose stimulus, therefore, causing a reduced insulin secretory capacity or delayed response (Brissova et al., 2006). This may help to explain the observed defects in the insulin secretory capacity of islets transplanted beneath the kidney capsule (Shi and Taljedal, 1996). In support of the suggested correlation between impaired glucose tolerance and islet vascularisation; a recent study by Christoffersson and colleagues demonstrated that mice receiving islets transplanted intraportally had both impaired glucose tolerance and suboptimal islet vascularisation, whereas islets transplanted to striated muscle had an endocrine vasculature that was restored to that of native pancreatic islets, correlating with similar glucose tolerance of recipient mice to non-diabetic non-transplanted control mice (Christoffersson et al., 2010). It should be noted that in our study, despite significantly enhancing the EC density within the islet endocrine tissue, MSCs did not completely restore the vasculature to the extent seen in the native pancreas (quantified as shown in Figure 3.10), which may help to explain why the presence of MSCs improves the rate and number of mice attaining normoglycaemia, but does not increase functionality after hyperglycaemia is reversed.

The rate of revascularisation of the islet graft is thought to have a major influence on graft survival and function and on the reversal of hyperglycaemia (Brissova and Powers, 2008). Conversely, hyperglycaemia has been associated with the delayed expression of angiogenic factors in islet grafts when compared to islet grafts in normoglycaemic recipients (Vasir et al., 2000). Thus, it seems likely that hyperglycaemia itself can impair the rate at which islet grafts revascularise. However, hyperglycaemia is not associated with any detrimental effect on the overall extent of islet revascularisation (Mendola et al., 1994, Mattsson et al., 2003). Co-

transplantation of kidney MSCs improved the overall extent to which islets revascularise at one month, consistent with previous observations in which MSCs derived from bone marrow (Figliuzzi et al., 2009, Ito et al., 2010) or pancreas (Sordi et al., 2010) have also been reported to increase the EC density of transplanted islets. Additionally, co-transplantation of kidney MSCs was also shown to increase the rate at which islets revascularise (as detailed in Chapter Five).

MSCs may influence angiogenesis through several mechanisms. They secrete a range of angiogenic factors, including vascular endothelial growth factor, IL-6, IL-8, hepatocyte growth factor and platelet-derived growth factor (Park et al., 2009, Sordi et al., 2010, Ito et al., 2010, Golocheikine et al., 2010), which are known to enhance islet revascularisation (Brissova et al., 2006, Cabric et al., 2010, Movahedi et al., 2008). In addition, MSCs secrete matrix metalloproteases (Ding et al., 2009), which facilitate the migration of host-derived ECs into the islets (Johansson et al., 2008) by degrading the extracellular matrix (ECM) (Ghajar et al., 2006, Potapova et al., 2007). Our morphological studies suggest that MSCs may also influence revascularisation and graft function by modulating the morphology of the graft. MSC co-transplantation had a profound impact on the remodeling process that occurs after transplantation, inducing a graft morphology that more closely represents that of islets in the endogenous pancreas, rather than the amorphous endocrine mass that formed at the graft site in islet-alone recipients, as reported previously for islets transplanted beneath the kidney capsule (Davalli et al., 1996, Biarnes et al., 2002). The MSC induced changes in graft morphology were associated with an altered distribution of ECs. Islet + MSC grafts showed a distribution of ECs throughout the endocrine tissue, similar to that of islets in the pancreas, whereas the islet-alone grafts showed a largely peripheral distribution of ECs, with large areas of endocrine tissue lacking ECs. The preferential localisation of ECs in the endocrine tissue of islet + MSC grafts is likely to be beneficial for graft function, as the endocrine cells rely on their oxygen supply mainly from blood supplied by capillaries in the endocrine tissue itself (Mattsson et al., 2002b). The enhanced vascularisation of the smaller endocrine aggregates of MSC co-transplanted mice is in accordance with previous studies demonstrating that the revascularisation of smaller islets after transplantation is more efficient than that of

larger islets (Kampf et al., 2006), an effect that may be amplified by the large aggregates formed in our islet-alone grafts.

Due to the heterogeneity of EC phenotype (Carmeliet, 2003), two separate EC markers were utilized to confirm the superior endocrine vascularisation of islet + MSC grafts. It is noteworthy that although the endocrine EC density was comparable with both markers; there were differences in the density of CD34+ and CD31+ ECs in the non-endocrine component of the islet + MSC grafts. The higher density of CD31+ ECs (although still very low) may reflect a subset of ECs with a more proliferative phenotype, as CD31 has been shown to be expressed by proliferating ECs (Nickoloff, 1993), whereas some studies have indicated that CD34 may be down-regulated in proliferating ECs (Fina et al., 1990). We cannot rule out the possibility that the CD31+ cells represent infiltrating monocytes or neutrophils, as these cellular subsets also express the CD31 antigen (Muller et al., 2002a). It is also possible that a small number of MSCs transdifferentiate into ECs, which could potentially explain the presence of this more active CD34-CD31+ subset of ECs at this time point. Indeed, a recent study by Ito and colleagues demonstrated that a small number of bone marrow MSCs were able to transdifferentiate into ECs at the islet graft site (Ito et al., 2010).

The re-establishment of a blood supply is of obvious importance for the survival and function of the islets, but it is also clear that paracrine interactions between β -cells and ECs are important in maintaining β -cell function (Linn et al., 2003b, Lai et al., 2005, Brissova et al., 2006, Johansson et al., 2009a, Olerud et al., 2011). The improved recovery of these interactions in the islet + MSC grafts may account for some beneficial effects of the MSC co-transplant in our model. Another striking observation from our analysis of EC distribution in the grafts was the low vascular density in the non-endocrine tissue surrounding the islets in the islet + MSC grafts, when compared with islet-alone grafts, in which the surrounding non-endocrine parenchyma contained large numbers of ECs, as reported previously (Mattsson et al., 2003, Olsson and Carlsson, 2005, Lau and Carlsson, 2009). These observations are consistent with earlier reports that transplanted islets induce increased vascularisation of the surrounding tissue to compensate for their low vascular density (Mattsson et al.,

2002b). The MSC-dependent increase in islet EC density observed by us may negate the requirement for a compensatory increase in revascularisation of the adjacent non-endocrine tissue. The MSC-induced alterations in graft morphology are also likely to influence graft survival and function independently of the rate or extent of revascularisation. A recent study using encapsulated islets, which are unable to revascularise, demonstrated improved transplantation outcomes from grafting of aggregates of smaller islets rather than of larger intact islets, consistent with an anatomical effect on function that is independent of revascularisation or EC density (O'Sullivan et al., 2010). Other studies have also demonstrated benefits of transplanting small islets, as opposed to larger islets, in terms of a islet revascularisation (Kampf et al., 2006) and it has also been shown that small islets have a higher oxygen consumption rate than larger islets, which has been shown to have a positive correlation with glucose-stimulated insulin secretion (Sweet et al., 2002, MacGregor et al., 2006).

It is well established that islet organisation influences function by facilitating the numerous interactions between the islet cells (Carvell et al., 2007) that are required for normal insulin secretion (Bosco et al., 1989, Hauge-Evans et al., 2009). The loss of organized islet anatomy in the islet-alone grafts, with α - and δ -cells distributed throughout the large endocrine aggregates is therefore likely to result in impaired function. Conversely, the MSC-dependent maintenance of normal islet size and organisation may contribute to the improved outcomes in the islet + MSC co-transplants. In particular, there is evidence to suggest that homologous contact between rodent β -cells is beneficial for their function (Bergsten et al., 1994, Bosco et al., 1989, Hauge-Evans et al., 1999, Luther et al., 2006, King et al., 2007), in contrast to heterologous contact with endocrine non β -cells, which appears to be less important (Bosco et al., 1989, King et al., 2007). Thus, the maintenance of islet architecture in the grafts of MSC co-transplanted mice favours homologous interactions between β -cells (Wojtusciszyn et al., 2008), which is likely to improve the synchronisation of individual β -cell activity. It is also of note that the orientation of blood flow is likely to have important functional implications for the regulation of blood glucose, as it determines the extent to which secreted hormones influence the different endocrine cell types within the islets. In a recent study by Nyman and colleagues, it was shown

that the predominant pattern of blood flow in endogenous islets was, ‘inner-outer’, with the β -cells being perfused before the α - and δ -cells (Nyman et al., 2008), in accordance with a number of other studies (Samols et al., 1988, Stagner and Samols, 1992, Menger et al., 1994). Thus, the disorganised islet architecture of islet-alone grafts coupled with the altered EC distribution and density is likely to disrupt the typical communication between endocrine cells via the intraislet vascular system, in addition to the disturbances in intercellular paracrine communication.

There was no evidence of MSCs remaining in the islet + MSC grafts at one month, although the presence of α -SMA⁺ cells at three days post transplantation, indicated that MSCs were detectable in the immediate vicinity of the islets during the initial days after transplantation. The non-endocrine component of the graft at one month appeared to be largely composed of collagen fibres, suggesting that MSCs may have secreted these ECM components themselves or transdifferentiated into a cell type, such as fibroblasts (Abraham et al., 2007) or chondrocytes (Nakata et al., 1992), with the capacity to do so. Interestingly, in this context, Jalili et al recently reported that a collagen matrix is beneficial to islet graft function (Jalili et al., 2011), supporting a number of previous studies indicating the importance of ECM support for optimal islet function and survival (Rosenberg et al., 1999, Thomas et al., 1999, Bosco et al., 2000, Hammar et al., 2004, Pinkse et al., 2006).

In summary, this study demonstrated that co-transplantation of MSCs has a beneficial effect on the outcome of islet grafts for treatment of diabetic hyperglycaemia, confirming recent reports (Jacobson et al., 2008, Ding et al., 2009, Solari et al., 2009, Figliuzzi et al., 2009, Park et al., 2009, Longoni et al., 2010, Sordi et al., 2010, Ito et al., 2010, Berman et al., 2010, Li et al., 2010, Kim et al., 2011). These effects may be due in part to enhanced revascularisation, as has been previously suggested (Figliuzzi et al., 2009, Sordi et al., 2010, Ito et al., 2010). However, our results also suggest that MSC dependent effects on the anatomical remodeling of the graft may have a major effect on graft function by maintaining islet organisation and morphology. Thus, subsequent experiments in this thesis have been designed to address the importance of islet organisation and morphology for improved glycaemic control, in the absence of an additional ‘islet helper cell’, such as MSCs that clearly have a number of additional

therapeutic properties. Additionally, we have addressed the role MSCs play in improving islet transplantation outcome during the early post transplantation period, with a focus on their ability to enhance the rate, as well as the overall extent of islet revascularisation as demonstrated in the current study.

5 Chapter Five - Graft remodelling and function in the early post transplantation period

5.1 Introduction

In the clinical islet transplantation setting, the majority of recipients require at least two donor pancreata in order to achieve normoglycaemia (Shapiro et al., 2000, Ryan et al., 2005a, Vantyghem et al., 2009). A similar ratio is needed for experimental rodent models of diabetes, which can largely be explained by the extensive islet cell death that occurs during the immediate post transplantation period. Over half of the transplanted islets undergo cell death by apoptosis or necrosis, even in a syngeneic setting (Davalli et al., 1996, Biarnes et al., 2002), where there are no additional cellular stresses resulting from immunological rejection. Improved graft function and engraftment during the early post transplantation period is correlated with better long term transplantation outcome (Warnock and Rajotte, 1988, Kaufman et al., 1990, Keymeulen et al., 1992, Tobin et al., 1993, Merino et al., 1997, Raurell et al., 1999), further emphasizing the need for interventional strategies to improve islet function and viability during the initial days after transplantation.

The reasons for extensive β -cell death are multifactorial (Davalli et al., 1996, Biarnes et al., 2002, Miao et al., 2006, Emamaullee et al., 2007), with factors related to the islet enzymatic isolation process and disruption to the peri-insular basement membrane (Rosenberg et al., 1999), as well as the pro-inflammatory microenvironment (Alejandro et al., 1986, Nagata et al., 1990, Barshes et al., 2005, Montolio et al., 2007) to which the islets are implanted. The metabolic status of the recipient has also been shown to affect the survival of transplanted β -cells (Merino et al., 1997, Biarnes et al., 2002), with hyperglycaemia adversely affecting vascular engraftment (Vasir et al., 2000) and β -cell survival in the longer term (Biarnes et al., 2002). The islet isolation procedure disrupts the islet vasculature and therefore the transplanted islets are avascular during the immediate post transplantation period (Menger et al., 1989, Miao et al., 2006), where they have to function in a hypoxic microenvironment (Miao et al., 2006), until the revascularisation process is completed. Islets are highly metabolically active and rely on oxygen for their optimal

function. Hypoxia can also lead to central necrosis of both cultured and transplanted islets (Dionne et al., 1993, Moritz et al., 2002). In addition to the early challenges regarding islet cell survival, it is estimated that two thirds of islets transplanted clinically never even become functional (Ryan et al., 2002), with a number of experimental studies also indicating disturbances in islet function (Dionne et al., 1993, Shi and Taljedal, 1996, Shi et al., 2000, Mattsson et al., 2004, Lau et al., 2007). Strategies to enhance the rate of islet graft revascularisation, with associated improvements in β -cell function (Lai et al., 2005, Brissova et al., 2006, Johansson et al., 2009c) are clearly warranted to make better use of the donor islet material that is available.

Mesenchymal stem cells (MSCs) have a number of functional properties, making them ideal cellular candidates for improving islet graft function and survival during the early post transplantation period. MSCs have been shown to secrete a number of angiogenic and anti-apoptotic factors (Figliuzzi et al., 2009, Park et al., 2009, Boumaza et al., 2009, Sordi et al., 2010, Ito et al., 2010, Berman et al., 2010), which could potentially reduce islet cell death and/or improve the survival of donor ECs and enhance the rate of revascularisation. The functional properties of MSCs are known to be modulated by the microenvironment in which they reside. Thus, during the early post transplantation period, pro-inflammatory cytokines, such as IFN- γ , TNF- α and IL1- β are present in high concentrations (Alejandro et al., 1986, Nagata et al., 1990, Barshes et al., 2005, Montolio et al., 2007) and are thought to upregulate the immunosuppressive properties of MSCs (Ryan et al., 2007, Krampera et al., 2006). The majority of immune-mediated islet cell death is thought to be due to inflammatory reactions, which precede the initiation of allograft rejection by the adaptive immune system (Lai et al., 2009), indicating that the immunosuppressive properties of MSCs may contribute to improved transplantation outcome in a syngeneic, as well as allogeneic setting.

Hypoxia is also thought to stimulate the angiogenic potential of MSCs (Annabi et al., 2003, Hung et al., 2007b, Efimenko et al., 2011), indicating that the microenvironment surrounding the co-transplanted islets and MSCs during the initial post transplantation period is likely to provide the appropriate stimuli for MSC-

modulated improvements in vascular engraftment. Additionally, MSCs may exert beneficial effects on β -cell function by up-regulating vital β -cell specific genes such as PDX-1, as shown in two recent studies (Boumaza et al., 2009, Park et al., 2010). This is likely to be particularly important in the early post transplantation period, where hypoxia can adversely affect β -cell function by down-regulating insulin and PDX-1 genes (Dionne et al., 1993).

Our kidney derived MSCs were shown to improve glycaemic control over a one month monitoring period, which was associated with increased vascularisation of the graft tissue and profound effects on the remodelling process at one month (see chapter four). Therefore, the aims of this study were to: 1) investigate the extent to which MSC co-transplantation altered graft morphology and islet architecture in the early post transplantation period; and 2) examine whether MSCs increased the rate, as well as overall extent of islet revascularisation and to determine whether this correlates with graft function.

5.2 Methods

5.2.1 Minimal islets mass model and experimental groups

Streptozotocin-diabetic C56Bl/6 mice were used as recipients. The number of islets transplanted aimed to cured diabetes in only a proportion of diabetic recipients. 150 fresh islets was chosen as a minimal islet mass. Islet + MSC recipients were transplanted with 150 fresh islets and 25×10^4 P7-10 MSCs, as with the previous one month study. Animals were considered cured if their non-fasting blood glucose concentrations were lower than 11.1mmol/l at the end-point of the experiment. Two experimental groups were used in order to study the time-course of islet revascularisation and determine whether MSCs were exerting any effect on the process at either time-point; the first was a three day study in which mice were monitored every day for three days after transplantation, at which point the grafts were harvested. The second group was for a seven day study, in which mice were monitored every other day for seven days and the grafts removed for histological analysis.

5.2.2 Evaluation of graft morphology and vascular density

From each animal three to six tissue sections from different regions of the graft were evaluated for total endocrine area and vascular density. The extent of islet fusion was evaluated by measuring the number and area of individual endocrine aggregates, as previously described in detail (see section 4.2.3). The area of endocrine tissue occupied by erythrocyte lakes was measured using image J software (<http://rsbweb.nih.gov/ij/>), on the same sections used to quantify total endocrine area and islet fusion (immunostained with insulin). Consecutive tissue sections were stained with Haematoxylin and Eosin for a clearer indication of cellular morphology and the presence of anuclear cells was indicative of erythrocytes.

5.2.3 TUNEL staining

The Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) method used terminal deoxynucleotidyl transferase to catalyze the polymerization of residues of digoxigenin-nucleotide to free 3'OH DNA strand breaks. The sections were deparaffinised and rehydrated as described (see section 2.9.5). Sections were washed in molecular biology grade water using DNase/RNase free tips and then with PBS. (DNase/RNase-free plastic ware was used throughout the protocol). Sections were placed in a humidified chamber and incubated with proteinase K solution (see reagents below) for 19 min at 37°C to permeabilise the tissue section. Sections were then washed twice with molecular biology grade water. A positive and negative control was carried out on sections consecutive to the tissue sample to confirm that the permeabilisation and labelling reaction had worked. A positive control was generated by treating a section with 1:50 TACS-nuclease diluted in TACS-nuclease buffer for 30 min at 37°C. Slides were immersed in PBS to stop the reaction. Endogenous peroxidase activity was quenched using 1:9 hydrogen peroxidase diluted in PBS for 5min at room temperature. Sections were then washed for 1 min in water, 1 min in PBS and 5 min in TdT labelling buffer (see reagents below). Tissue sections were incubated with the labelling reaction mix (see reagents below) at 37°C for 2 hr, after which slides were transferred to TdT stop buffer for 10min at room temperature. Sections were washed in PBS twice for 2 min before incubating with streptavidin-HRP solution (see reagents below) for 10 min at room temperature. Lastly tissue

sections were developed using DAB solution and the reaction monitored under a light microscope, before washing in water and mounting as normal (see section 2.9.5).

5.2.4 Reagents

Proteinase K solution

<u>Reagent</u>	<u>For 50µl</u>
Deionised water	1µl
Proteinase K (provided in the kit)	49µl

Proteinase K solution was made up fresh for each TUNEL assay.

Quenching solution

<u>Reagent</u>	<u>For 50ml</u>
Methanol	45ml
H₂O₂ (30% (w/v))	5ml

Quenching solution was made up fresh and used at room temperature.

1x TdT labelling buffer

10x TdT labelling buffer (provided in the kit) was diluted 1:10 using molecular biology grade water (50µl per section required) and stored at room temperature until use.

Labelling reaction mix

<u>Reagent</u>	<u>For 50µl</u>
TdT – dNTP	1µl
50x cation stock (Co²⁺)	1µl
TdT enzyme	1µl
1X TdT labelling buffer (as above)	47µl

The TdT – dNTP mix was thawed at room temperature and then place on ice. The labelling reaction mix was prepared immediately before use and keep on ice. Abbreviations: TdT, Terminal deoxynucleotidyl Transferase

Streptavidin-HRP Solution

<u>Reagent</u>	<u>For 50µl</u>
1xPBS	49µl
Streptavidin-HRP	1µl

Streptavidin-HRP Solution was made and stored at room temperature until use.

DAB solution

<u>Reagent</u>	<u>For 50ml</u>
1X PBS	50ml
DAB	250µl
30% H₂O₂	50µl

The DAB provided in the kit was thawed in a water bath pre-heated to 37°C, no more than 20 min before use.

TACs nuclease and buffer

<u>Reagent</u>	<u>For 50µl</u>
TACS-Nuclease Buffer	49µl
TACS-Nuclease	1µl

TACs nuclease was made up fresh and kept on ice.

5.3 Results

The effect of MSC co-transplantation on islet graft function and engraftment during the early post transplantation period was investigated over two separate time points. In the first set of experiments, mice were monitored for three days before harvesting the grafts for histological analysis of morphological and vascular engraftment. In the second set of experiments, mice were monitored over a seven day period and the grafts harvested and analysed in the same way.

5.3.1 Graft function over a three day monitoring period

Blood glucose concentrations were monitored every day during the immediate post transplantation period (days 1-3). The average blood glucose concentrations of mice transplanted with islets alone were slightly higher than those of islet + MSC

recipients; however this did not reach statistical significance at any time (Figure 5.1a). After three days, 67 percent of mice transplanted with islets alone had not been cured, compared to 40 percent of mice co-transplanted with islets + MSCs (Figure 5.1b). There were no differences in the weights of mice in both transplant groups on day 0 (22.0 ± 1.1 and 21.9 ± 1.1 g for islet-alone and islet + MSC recipients respectively, $n=5-6$, $p = 0.94$, Student's t test) or on day three (22.4 ± 1.2 and 21.6 ± 0.6 g, $n=5-6$, $p = 0.60$, Student's t test).

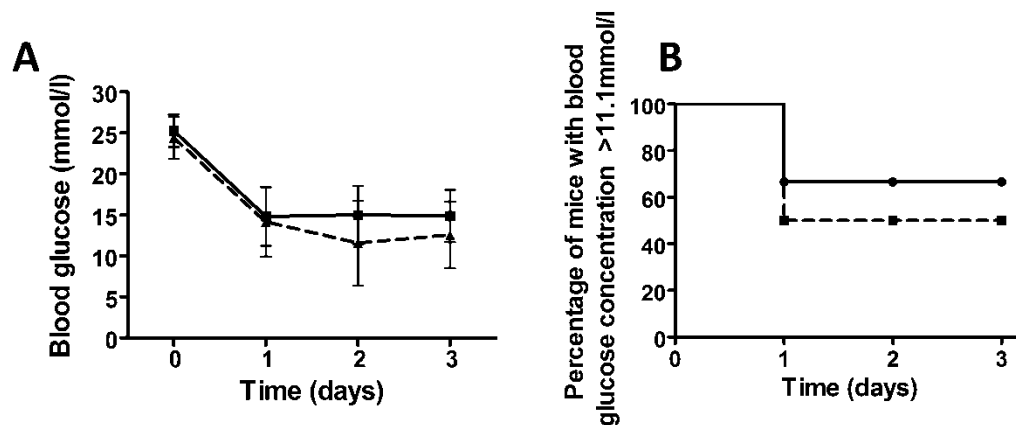


Figure 5.1 Graft function three day study. **A.** Blood glucose concentrations of mice transplanted with islets-alone (continuous line) or those co-transplanted with islets + 25×10^4 kidney-derived MSCs (dashed line); $p > 0.05$, RM ANOVA with Bonferroni post hoc test, $n=5-6$. **B.** Percentage of mice remaining diabetic (blood glucose concentration >11.1 mmol/l) after transplantation, as in A, $p = 0.58$, Kaplan-Meier, $n=5-6$.

5.3.2 MSC localisation at three days post transplantation

At three days post transplantation, cells staining positively with α -SMA (a marker which has been shown to be expressed by murine MSCs (Meirelles et al., 2006)) antibodies could be seen surrounding the insulin-positive endocrine tissue in the grafts of islet + MSC recipients, strongly indicating the presence of co-transplanted MSCs in the immediate vicinity of the islets, as shown in Figure 5.2.

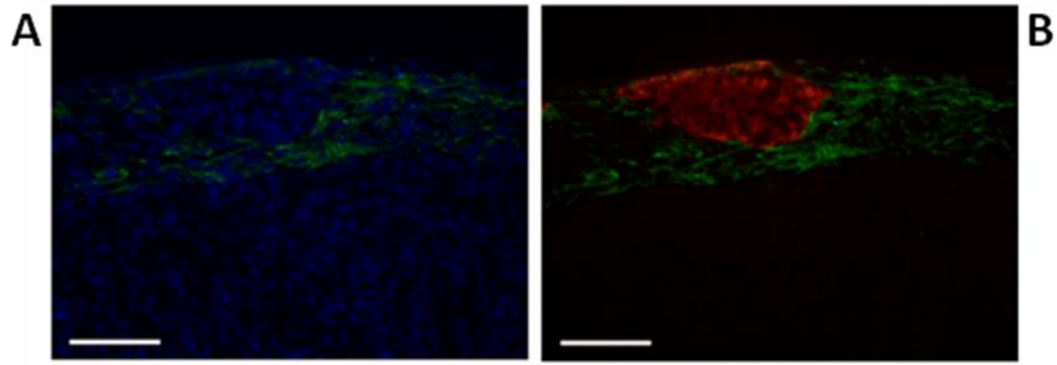


Figure 5.2 MSC localisation. A. Islet + MSC grafts, where α -SMA-positive cells (green), dapi (blue) can be seen surrounding the insulin-positive (red) endocrine tissue as in (b), images are representative of graft sections from four animals, original magnification x200, scale bars are 25 μ m.

5.3.3 Graft morphology at three days post transplantation

Figure 5.3 demonstrates the typical morphology of graft material retrieved at three days post transplantation. Insulin immunostaining of islet-alone grafts revealed that the islet fusion that is completed at one month post transplantation (see Figure 4.5) has not yet fully occurred, but that the individual islets that can be seen appear to be merging at this time point (Figure 5.3a). Individual islets can also be discerned in the grafts of islet + MSC recipients, but do not appear to be fusing with each other (Figure 5.3b). In the grafts of both transplant groups, there were clearly extensive signs of remodelling, with non-endocrine islet cores, surrounded by an apparently viable rim of insulin-positive endocrine tissue. Hematoxylin and Eosin staining shows that there are anuclear cells present within these islet cores, indicating the presence of erythrocytes (Figure 5.3c and d). There was no difference in the overall area of endocrine tissue occupied by erythrocyte lakes between transplant groups (8.82 ± 1.59 vs 21.87 ± 3.29 percent, $n=3$, $p = 0.24$, Student's t test, for islet-alone and islet + MSC grafts respectively). There was also no correlation between the average endocrine aggregate area per graft section and the percentage of endocrine tissue occupied by erythrocytes, however it was noted that they were absent in the smaller individual islets (all islets $<10,000\mu\text{m}^2$).

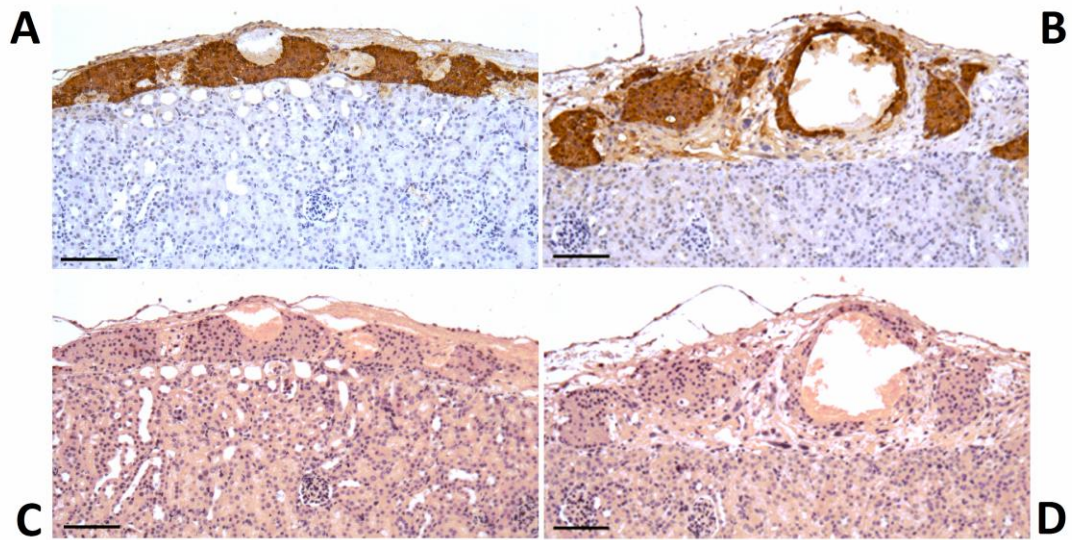


Figure 5.3 Graft morphology at three days post transplantation. A, C. Representative sections of islet-alone grafts and (b, d) islet + MSC grafts at three days post transplantation. Consecutive graft sections were stained with insulin antibodies (a, b) and Haematoxylin and Eosin (c, d), images are representative of three animals in each transplant group, original magnification x100, scale bars are 100 μ m.

Insulin immunostaining also revealed that there were regions within both islet-alone (Figure 5.4a) and islet + MSC grafts (Figure 5.4b) where the insulin-positive tissue was completely intact without central regions of the transplanted islet tissue lacking β -cells. There was considerable variation in the extent to which islets had fused in the grafts of mice transplanted with islets-alone. The area of non-endocrine tissue separating islets in the islet + MSC transplant group also showed a high degree of variability. Quantification of graft morphology showed that the total area of endocrine tissue per section was comparable in islet-alone and islet + MSC recipients (Figure 5.4c). The average area of individual endocrine aggregates was also similar between transplant groups (Figure 5.4d). In accordance, there were no differences in the total graft area (endocrine and non-endocrine tissue) between transplant groups ($173,093 \pm 57,149$ and $220,679 \pm 28,585 \mu\text{m}^2$ for islet-alone and islet + MSC grafts respectively, $p = 0.50$, $n=3$, Student's t test).

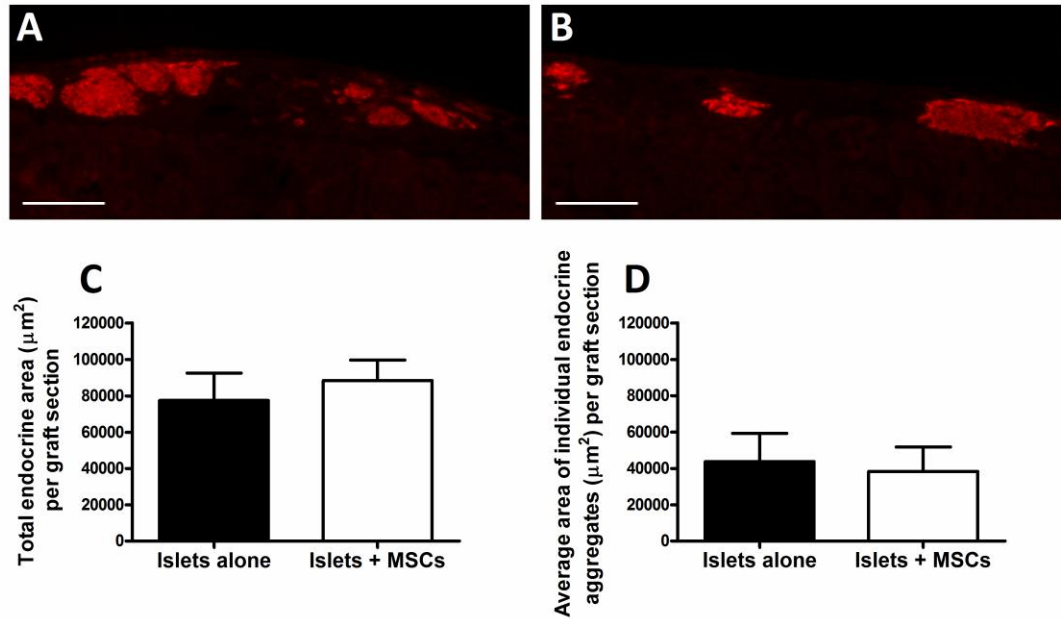


Figure 5.4 Extent of islet fusion at three days post transplantation. Representative sections of islet-alone grafts (a) and islet + MSC grafts (b) at three days post transplantation, immunostained with insulin antibodies, original magnification $\times 100$, scale bars are $100\mu\text{m}$. **C.** Total endocrine area per graft section, $n=3$ animals per transplant group, $p = 0.84$, Student's t test. **D.** Average individual endocrine aggregate area per graft section, $n=3$ animals per transplant group, $p = 0.64$, Student's t test.

5.3.4 Apoptosis by TUNEL

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was optimised for detecting apoptotic cells in islet graft sections. The number of apoptotic cells was exceptionally low in the graft sections analysed from islet-alone recipients at three days post transplantation, with only one TUNEL-positive nucleus seen throughout the graft section in one animal (Figure 5.5a) and no apoptotic nuclei in the second animal. Consecutive positive control (Figure 5.5b) and negative control (Figure 5.5c) tissue sections confirm that the protocol works, with no signs of any background staining, which may would potentially lead to false positive results. We did not carry out any further investigations of apoptotic cell death in the three day grafts of islet + MSC recipients, given that there were so few TUNEL-positive nuclei observed in the islet-alone control mice and therefore nothing for MSC co-transplantation to improve upon at this time point.

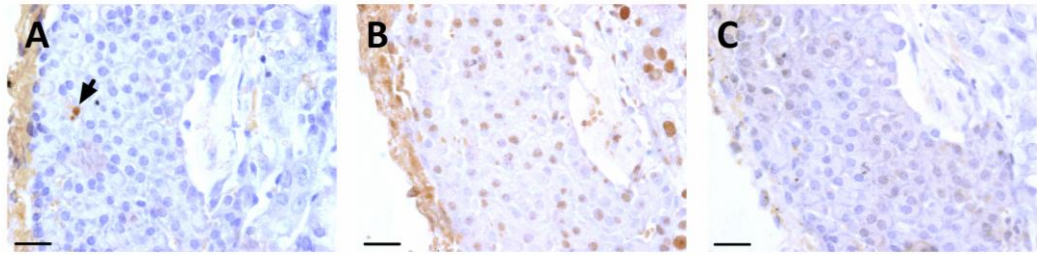


Figure 5.5 TUNEL staining. Micrographs showing an islet-alone graft three days after transplantation to a STZ-diabetic recipient. **A.** Section stained for TUNEL-positive cells. **B.** Consecutive section positive control **C.** Consecutive section negative control. Images are representative of sections from two mice transplanted with islet-alone grafts. Original magnification x400, scale bars are 25 μ m.

5.3.5 Graft composition at three days post transplantation

Immunostaining for glucagon positive α -cells indicated that they were mostly located at the periphery of individual islets in the grafts of islet-alone (Figure 5.6a) and islet + MSC recipients (Figure 5.6b). The distribution of somatostatin positive δ -cells was similar to that for α -cells in islet-alone (Figure 5.6c) and islet + MSC (Figure 5.6d) grafts. Quantification of both α - and δ -cells revealed no differences in the percentage of these cell types within the grafts of either transplant groups (Figure 5.6e and f respectively).

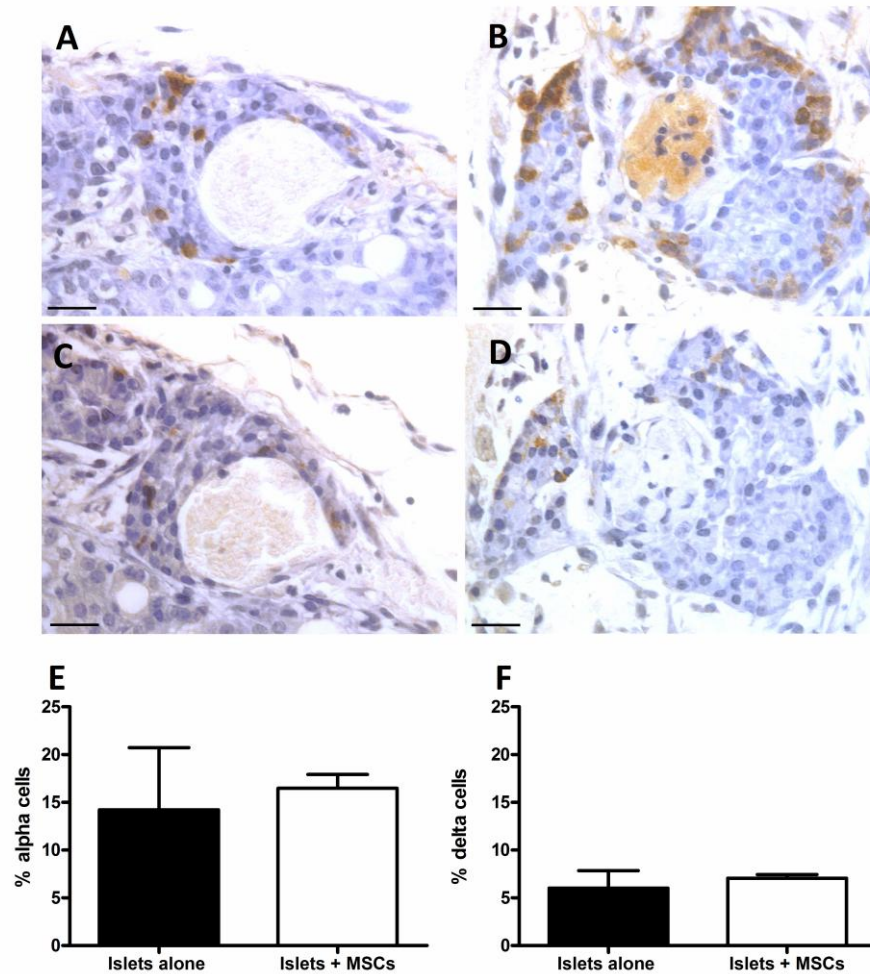


Figure 5.6 Graft composition at three days post transplantation. Distribution of glucagon-positive α -cells and somatostatin-positive δ -cells. At three days post transplantation, consecutive sections were stained with glucagon (a, b) or somatostatin antibodies (c, d), in grafts consisting of islets alone (a, c) or islets + 25×10^4 kidney-derived MSCs (b, d), images are representative of sections from three different animals in each transplant group, original magnification $\times 400$, scale bars are $25\mu\text{m}$. **E.** Percentage of α -cells in graft sections; $n=3$ animals per transplant group, $p = 0.75$, Student's t test. **F.** Percentage of δ -cells in graft sections; $n=3$ animals per transplant group, $p = 0.61$, Student's t test.

5.3.6 Vascular density of islet grafts at three days post transplantation

CD34 and CD31 antibodies were used to immunostain ECs in consecutive islet graft sections. There were very few CD34+ ECs within both the endocrine and non-endocrine tissue of islet-alone recipients (Figure 5.7a). The graft material of islet + MSC recipients showed a similarly low number of CD34+ ECs in both components of the graft (Figure 5.7b). Immunostaining with CD31 antibodies also showed that the endocrine tissue in islet-alone recipients had a very low density of ECs. However, there was clearly a greater density of CD31+ ECs than those staining positively with

CD34 antibodies, in the non-endocrine tissue surrounding the islets (Figure 5.7c). Similarly, in islet + MSC grafts, there was a high density of CD31+ ECs surrounding the islets (Figure 5.7d), in contrast with the virtually absent expression of CD34+ ECs. Quantification of CD34+ and CD31+ ECs showed that there was differential expression of CD31+ and CD34+ ECs, in the non-endocrine tissue surrounding the islets for both transplant groups, with a markedly higher number of CD31+ ECs present (Figure 5.7e).

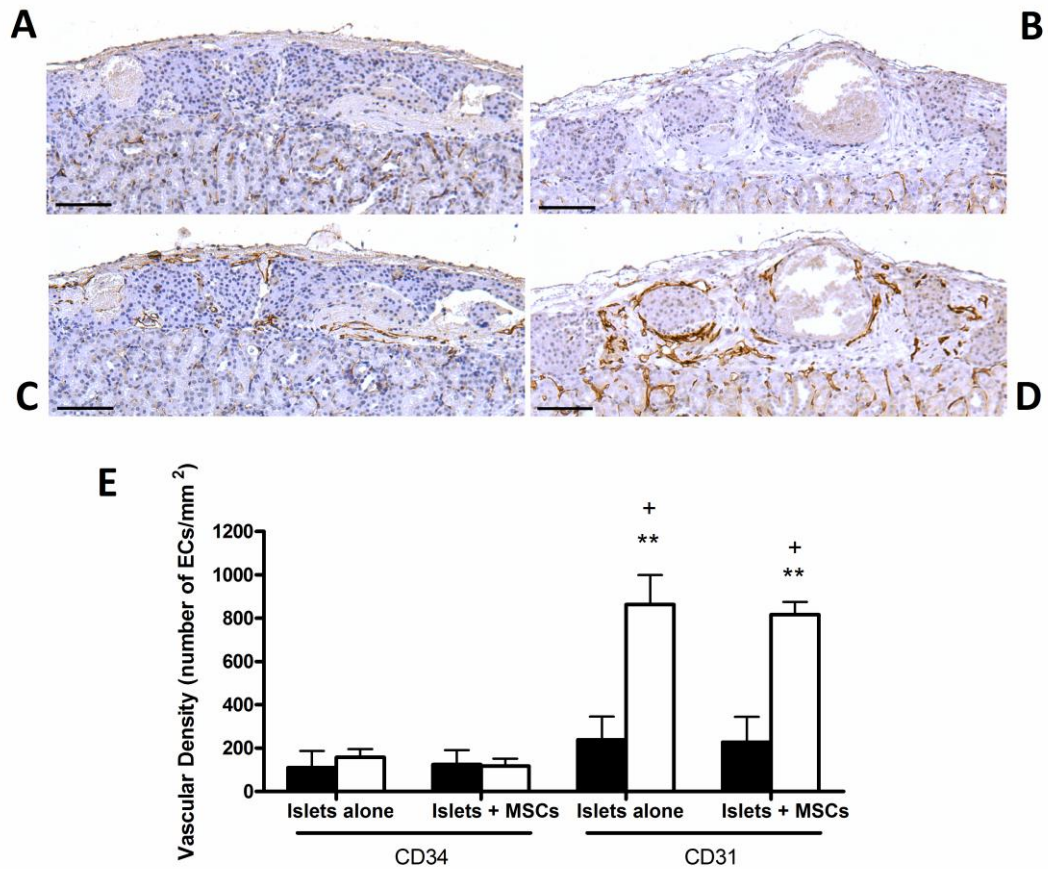


Figure 5.7 Differential expression of EC markers in the non-endocrine tissue of islet grafts at three days post transplantation. Consecutive sections were stained with CD34 (a, b) and CD31 (c, d) antibodies in grafts consisting of islets-alone (a, c) or islets + MSCs (b, d) and are representative of sections from four different animals in each group, original magnification x100, scale bars are 100µm. **E.** Vascular density of endocrine components (black bars) and non-endocrine components (white bars) of three day grafts consisting of islets-alone or islets + 25×10^4 kidney-derived MSCs, $^+p < 0.05$ vs endocrine tissue within same transplant group and marker, $^{**}p < 0.01$ vs CD34 immunostained islet + MSC grafts within the same tissue type, n=4, two-way ANOVA with Bonferroni post hoc test.

5.3.7 Graft function over a seven day monitoring period

Blood glucose concentrations were monitored over a seven day time period in a second group of animals to determine whether there were any differences in graft function and/or engraftment when grafts were retrieved at an intermediate time point between the current three day study and the previous one month study (see Chapter Four). Co-transplantation of islets with MSCs had no beneficial effect on glycaemic control in this study as shown in Figure 5.8a, with average blood glucose concentrations below 13mmol/l for both transplant groups at day 7. Thus, compared with the one month study (see Figure 4.3), the minimal islet mass model failed, with the glycaemic control in islet-alone recipients being better than expected. After seven days, 50% of islet-alone mice had cured (cured defined as blood glucose <11.1mmol/l), compared with 80% of MSC co-transplanted mice ($p = 0.87$, Kaplan-Meier, $n=5-6$). There were no significant differences in the starting weight of the mice in either transplant group (Figure 5.8b). However, there was a trend towards control islet-alone mice in this seven day study having a lower starting weight than those in the previous one month study (Figure 5.8c), indicating that 150 fresh islets is not a suitable minimal islet mass for the lighter mice (average weight at time of transplantation; 22.1 ± 1.1 g, $n=6$) in the current study. The graft-bearing kidney of all cured mice in each transplant group was removed on day seven. All nephrectomised mice reverted to hyperglycaemia within two days (Figure 5.8d), strongly indicating that the maintenance of normoglycaemia was not due to endogenous β -cell regeneration in the pancreas.

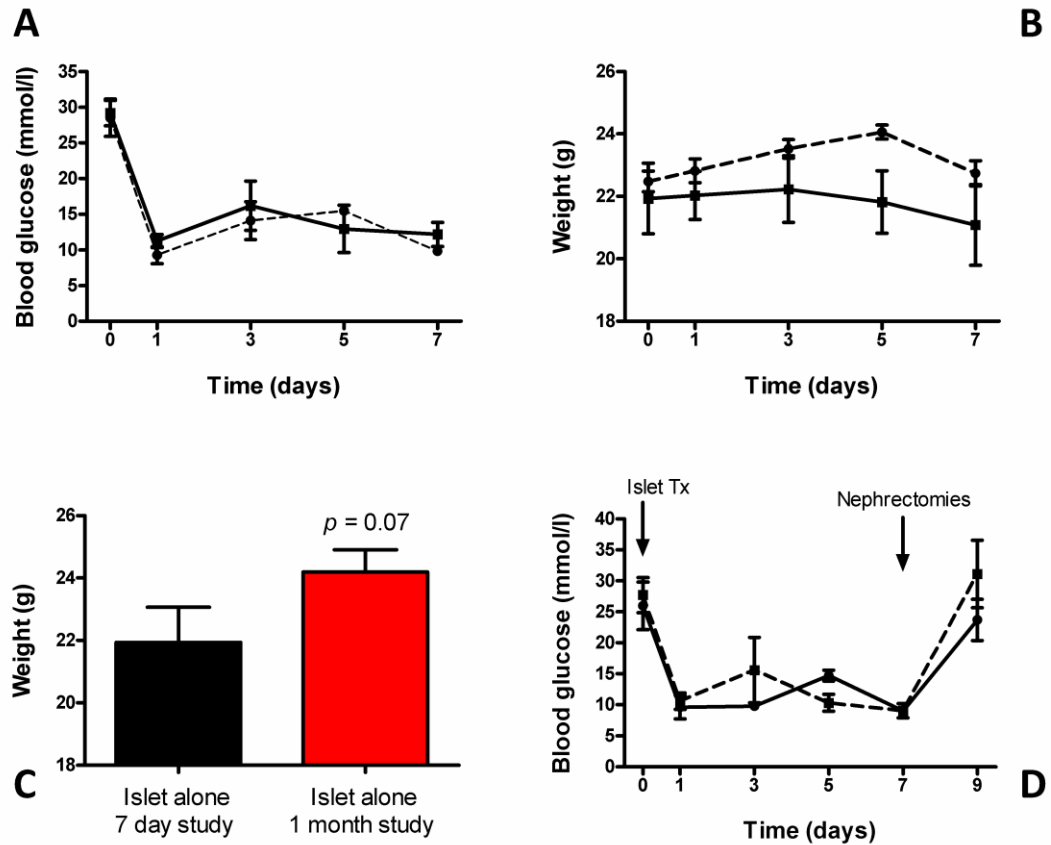


Figure 5.8 Graft function seven day study. **A.** Blood glucose concentrations of mice transplanted with islets alone (continuous line) or co-transplanted with islets + 25×10^4 kidney-derived MSCs (dashed line); $p > 0.05$ (RM ANOVA with Bonferroni post hoc test, $n=5-6$). **B.** Weight of mice transplanted with islets alone or co-transplanted with islets + MSCs ($n=5-6$). **C.** Weight of control islet-alone mice in the current seven day study and control islet-alone mice in the one month study, $p = 0.07$ vs islet-alone previous study, Student's t test, $n = 6-13$. **D.** Blood glucose concentrations of cured mice only, after islet transplantation (day 0) and after removal of the graft bearing kidney at seven days post transplantation ($n=3$ in both transplant groups).

5.3.8 Graft morphology at seven days post transplantation

The graft morphology at seven days was variable between animals, however, in general it could be seen that the graft tissue in islet-alone recipients consisted largely of fused islets forming an amorphous mass of insulin-positive endocrine tissue, as shown in (Figure 5.9a). Individual islets could still be seen in the islet + MSC grafts (Figure 5.9b), with signs of remodelling and the persistence of erythrocyte lakes evident in the grafts of both transplant groups. There were no differences in the total endocrine area (immunostained for insulin) per graft section, between transplant groups (Figure 5.9c). Although there were signs that the overall graft morphology was not the same, no differences in the extent of islet fusion (Figure 5.9d) were

determined using the quantification method previously described (see Chapter Four). The total graft area (endocrine and non-endocrine tissue) was similar between transplant groups ($130,811 \pm 13,915$ vs $166,305 \pm 32,232 \mu\text{m}^2$, $p = 0.35$, $n=4$, Student's t test, for islet-alone and islet + MSC recipients respectively).

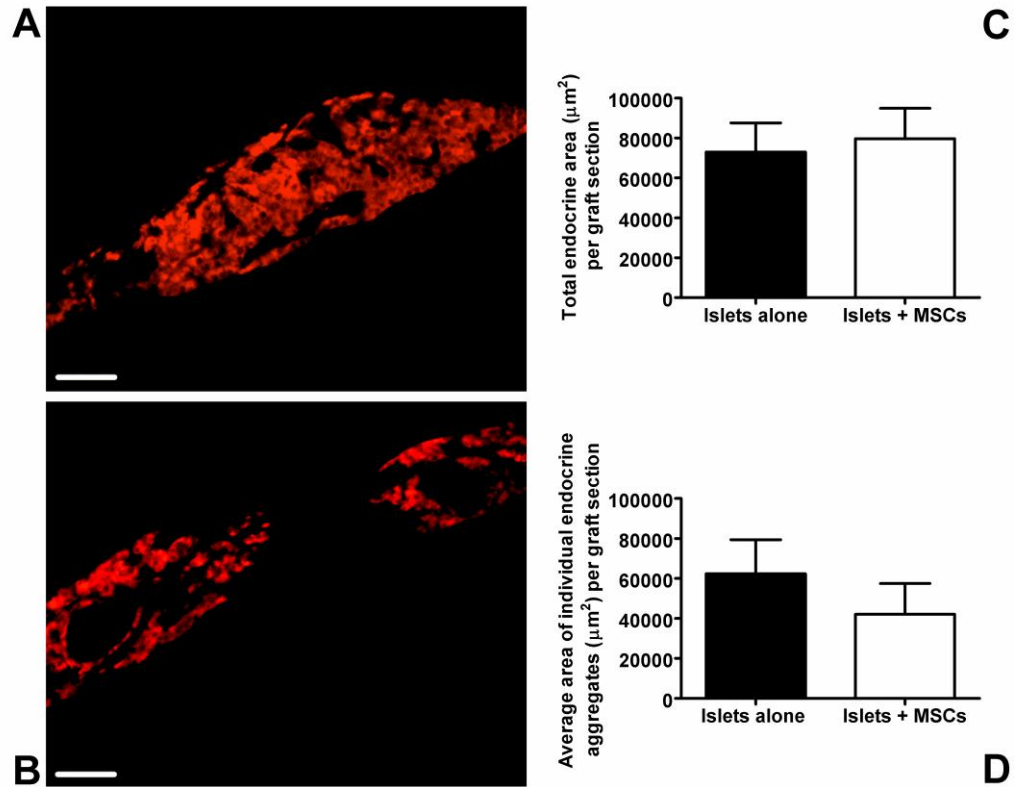


Figure 5.9 Graft morphology at seven days post transplantation. Representative sections of islet-alone grafts (a) and islet + MSC grafts (b) at seven days post transplantation, immunostained with insulin antibodies, original magnification $\times 100$, scale bars are $50 \mu\text{m}$. **C.** Total endocrine area per graft section, $n=4$ animals per transplant group, $p = 0.76$, Student's t test. **D.** Average individual endocrine aggregate area per graft section, $n=4$ animals per transplant group, $p = 0.41$, Student's t test.

5.3.9 Vascular density of islet grafts at seven days post transplantation

There was clearly a difference in the distribution of CD34+ ECs at seven days post transplantation in both transplant groups, compared with that seen at three days post transplantation, which was far more pronounced in the grafts of islet + MSC recipients. In islet-alone recipients CD34+ ECs were present throughout the endocrine tissue, albeit in very low numbers (Figure 5.10a), indicating that the revascularisation process had been initiated. In the grafts of islet + MSC recipients, CD34+ ECs were present throughout the endocrine tissue in large numbers (Figure 5.10b). The vascular

density of the endocrine tissue was greater in the grafts of MSC co-transplanted mice, compared to islet-alone recipients. There was a markedly higher vascularisation of the surrounding non-endocrine tissue compared to the endocrine tissue observed in islet-alone grafts (Figure 5.10c).

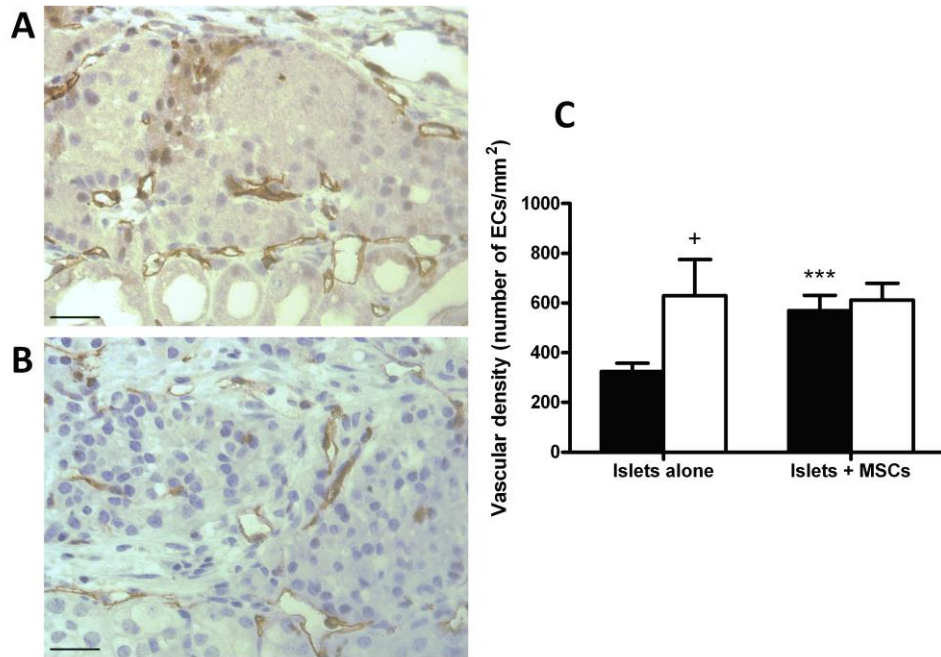


Figure 5.10 Vascular density at seven days post transplantation. CD34 antibodies were used to immunostain microvascular ECs in the grafts of islet-alone (a) and islet + MSC recipients (b), original magnification x400, scale bars are 25µm. C. Vascular density of endocrine components (black bars) and non-endocrine components (white bars) in seven day grafts consisting of islets alone or islets + 25×10^4 kidney-derived MSCs, $n=4$, $***p < 0.001$ vs the endocrine components of the grafts from islet-alone recipients immunostained with CD34 antibodies, $^+p < 0.05$ vs endocrine tissue in same transplant group and marker, two-way ANOVA with Bonferroni post hoc test.

5.3.10 Differential expression of endothelial cell markers within non-endocrine graft component at seven days post transplantation

Consecutive sections of seven day islet grafts consisting of islets alone or islets + MSCs were stained with CD34 and CD31 antibodies, to determine whether there was differential expression of these markers, as with the three day grafts. CD34+ ECs were present in low numbers within the endocrine tissue of seven day islet-alone grafts (Figure 5.11a), with smaller regions of endocrine tissue in the islet + MSC grafts devoid of any CD34+ ECs (Figure 5.11b), as shown previously (see Figure 5.10). There was a similar distribution of CD31+ ECs (compared to CD34+ ECs)

within the endocrine tissue of islet-alone recipients (Figure 5.11c) and islet + MSC recipients (Figure 5.11d), however quantification of both CD34+ and CD31+ ECs, showed that there was a markedly higher number of CD31+ ECs in the non-endocrine tissue of both transplant groups, compared to the endocrine tissue, as shown in Figure 5.11e. The additional use of CD31 as an EC marker confirmed the enhanced vascularisation of the endocrine tissue in islet + MSC grafts, compared to islet-alone grafts, with a significantly higher number of both CD34+ and CD31+ ECs found (Figure 5.11e).

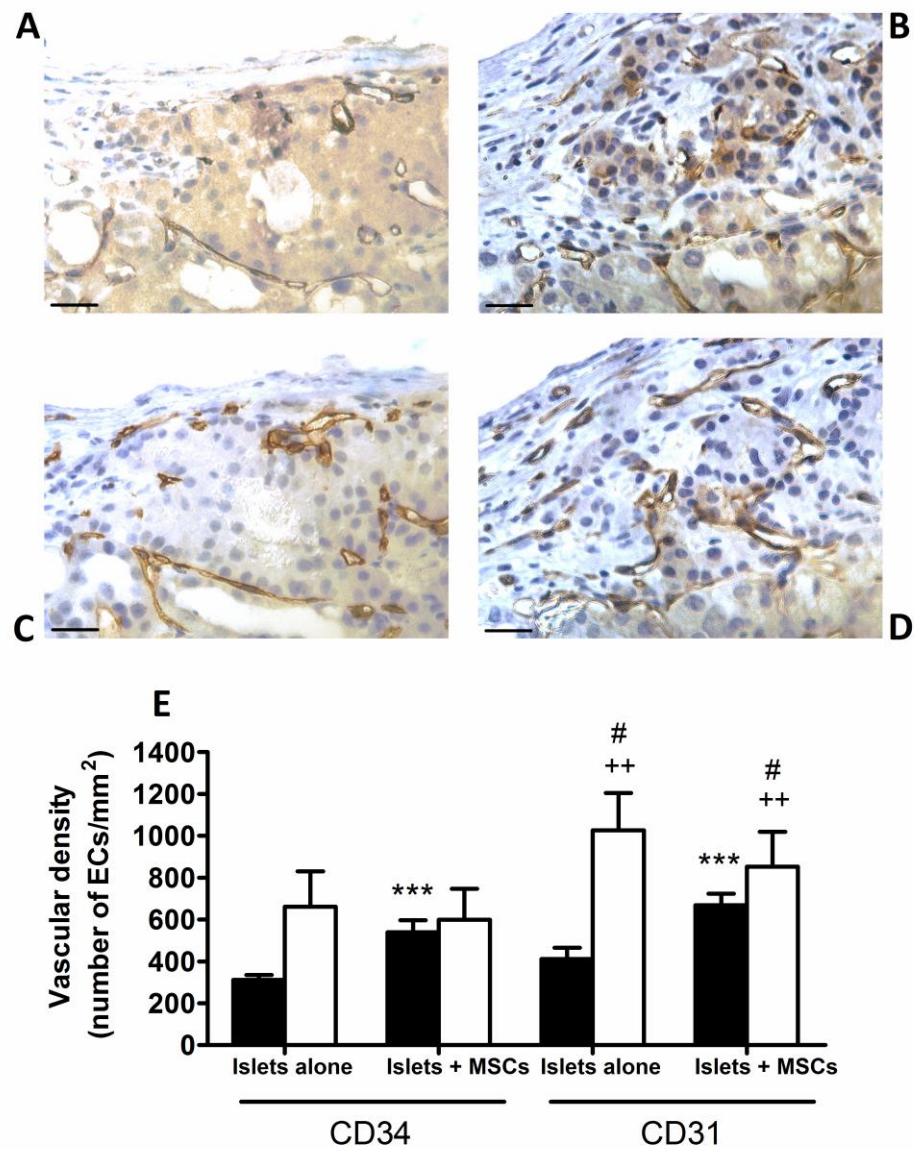


Figure 5.11 Differential expression of EC markers in the non-endocrine tissue of islet grafts at seven days post transplantation. Consecutive sections were stained with CD34 (a, b) and CD31 (c, d) antibodies in grafts consisting of islets-alone (a, c) or islets + MSCs (b, d) and are representative of sections from four different animals in each group, original magnification x400, scale bars are 25μm. **E.** Vascular density of endocrine components (black bars) and non-endocrine components (white bars) of seven day grafts consisting of islets alone or islets + 25×10^4 kidney-derived MSCs, $^{++}p < 0.01$ vs CD34 immunostained tissue sections within non-endocrine tissue in same transplant group, $^{***}p < 0.001$ vs islet alone sections in same tissue type, with same marker, $n=4$, $^{\#}p < 0.05$ vs endocrine tissue within same marker and transplant group, two-way ANOVA with Holm-Sidak post hoc test.

5.4 Discussion

A syngeneic minimal islet mass model was used to determine the effects of kidney-derived MSCs on graft function and engraftment during the early post transplantation

period. A number of reports suggest that MSCs secrete trophic factors that might improve vascular engraftment and/or islet function during the immediate post transplantation period (Figliuzzi et al., 2009, Park et al., 2009, Boumaza et al., 2009, Sordi et al., 2010, Ito et al., 2010), when it is most likely that MSCs would persist at the graft site.

Islets are known to be particularly vulnerable during the first few days after transplantation (Davalli et al., 1996, Biarnes et al., 2002, Miao et al., 2006, Emamaullee et al., 2007), due to hypoxic stresses (Dionne et al., 1993, Miao et al., 2006), associated with the lack of vascularisation, as shown in this study and in support of previous findings from other groups (Menger et al., 1989, Miao et al., 2006). The transplanted islets are therefore dependent on the diffusion of oxygen, which is slow and contributes to the reduced insulin secretory capacity observed in transplanted islets (Dionne et al., 1993). Interestingly, MSCs are thought to retain their stem cell properties better when cultured in low oxygen tensions (Grayson et al., 2006), which can also reduce the capacity of MSCs to differentiate into other cell types (Hung et al., 2007b), suggesting that the situation may be similar *in vivo*. Indeed, positive immunostaining of islet + MSC graft sections at three days post transplantation with α -SMA antibodies (a marker which has previously been shown to be expressed by murine MSCs (Meirelles et al., 2006), strongly indicated the presence of co-transplanted MSCs in the immediate vicinity of the transplanted islets at this time point.

Recent studies have shown that the local microenvironment can affect the functional properties of MSCs. In particular, non-specific inflammatory signals such as IFN- γ can upregulate the immunosuppressive properties of MSCs (Ryan et al., 2007, Krampera et al., 2006), which could potentially reduce islet cell death associated with the proinflammatory microenvironment to which the islets are transplanted (Alejandro et al., 1986, Nagata et al., 1990, Barshes et al., 2005). It has also been shown that MSCs can increase their production of angiogenic factors, such as VEGF in response to hypoxia (Annabi et al., 2003, Hung et al., 2007b, Efimenko et al., 2011), thus suggesting that the transplanted MSCs may be able to enhance intraislet EC survival and/or increase the rate of revascularisation during the immediate post transplantation

period. Despite this, there was no beneficial influence of MSC co-transplantation on the vascularisation of the transplanted islets at three days, correlating with the lack of effect on graft function during this time.

There were extensive signs of remodelling during the immediate post transplantation period, with abnormal islet morphology and architecture observed in the grafts of mice in both transplant groups, in accordance with the findings of Morini and colleagues (Morini et al., 2007). The absence of infiltrating mononuclear cells within the islet cores suggests that necrosis has already occurred, if indeed this is contributing to any cell death in the first three days post transplantation, as has previously been reported by other groups (Davalli et al., 1996, Biarnes et al., 2002). The finding of anuclear cells within central parts of the transplanted endocrine tissue is indicative of the presence of erythrocyte lakes (Biarnes et al., 2002). In accordance with the study by Gray et al, there was no correlation between the presence of erythrocyte lakes and graft function. It has also been suggested that their presence may be correlated with engraftment and revascularisation (Biarnes et al., 2002), which is not contradicted in this study, as both graft vascularisation and the presence of erythrocyte lakes was comparable between transplant groups. An explanation for the presence of these 'erythrocyte filled cavities' may be that there are blood vessels within transplanted islets completely lacking an EC lining, due to the structural damage to intraislet capillaries, which occurs during the isolation procedure (Lukinius et al., 1995). To date, there have been no extensive studies investigating whether these blood filled structures can mature functionally with time to form endothelialised vessels. However, the results presented in this thesis are consistent with Lukinius and colleagues in the finding that their number decreases with time after transplantation. Apoptosis levels appear to be very low at three days, suggesting that the majority of islet cell death may well have already occurred at this time point, as has previously been shown with the finding that β -cell survival and mass, as well as insulin content declines between 1-3 days post transplantation (Davalli et al., 1996). Biarnés et al have also shown that erythrocyte lakes are present after the extensive β -cell apoptosis and necrosis had occurred, supporting the idea that the majority of islet cell death has already taken place at three days post transplantation (Biarnes et al., 2002).

MSC co-transplantation was previously shown to improve graft function, with associated effects on the engraftment process at one month post transplantation (see Chapter Four). Notably, there was a profound impact on the graft morphology, with reduced fusion between individual islets, resulting in a significantly lower average endocrine aggregate area per graft section. The fusion of individual islets has been recognised as a pathological feature of islet graft remodelling during the immediate post transplantation period (Davalli et al., 1996, Biarnes et al., 2002). Therefore, it is important to gain an understanding of how MSCs might be affecting this process and the time-course over which the fusion process typically occurs. At three days post transplantation, there were already signs of fusion between individual islets in the grafts of islet-alone recipients. For islet + MSC recipients, the graft morphology was variable. However, there were no signs of fusion between individual islets in any of the graft sections analyzed, despite islets being in close proximity to each other. Quantification of the extent of islet fusion revealed that there were no differences in either total endocrine area or the average endocrine aggregate area per graft section. Given that MSC co-transplantation significantly reduced the average endocrine aggregate area at one month, this indicates that there are MSC-dependent alterations in the remodelling process between three days and one month. Also in contrast to the observed graft morphology at one month, where there was a significantly higher area of non-endocrine tissue; there were no significant differences in the total graft area at three days, indicating that the amount of non-endocrine tissue within the islet + MSC grafts increases between three days and one month. There are a number of possible explanations for this. Firstly, due to the technical procedure used for the co-transplantations, the islet pellet was directly in front of and not necessarily mixed with the MSC pellet in the tubing. The migration of MSCs to islets and other sites of tissue injury have previously been reported (Barbash et al., 2003, Lee et al., 2006, Fox et al., 2007). Therefore, it may well be that more of the transplanted MSCs migrate to the immediate vicinity of the islet tissue after three days, thereby increasing the total graft area. Secondly, the non-endocrine tissue at one month was shown to be mostly acellular with positive van Geison staining indicating the presence of collagen fibres. MSCs have been shown to secrete collagen and other stromal components (Pittenger et al., 1999) and it is therefore likely that this process has not fully occurred during the early post transplantation period. Interestingly in this context, other studies have

shown that MSCs are able to withstand hypoxia-induced apoptosis and proliferate under such conditions (Hung et al., 2007b), indicating that the microenvironment to which they are transplanted is not likely to cause a decline in their number, in contrast to islet cells (Davalli et al., 1996, Biarnes et al., 2002, Miao et al., 2006) and ECs (Lai et al., 2005), which are susceptible to hypoxia-induced cell death. Notably and in accordance with the incomplete fusion seen at three days after transplantation for both transplant groups, as with previous reports (Morini et al., 2007); endocrine non- β -cells appeared to maintain their localisation at the periphery of individual islets. This suggests that the disorganised islet architecture seen at one month in the grafts of islet-alone recipients is related to the fusion of islets that occurs at a later point during the engraftment process.

The rate of islet graft revascularisation is thought to have a major influence on graft function and survival and on the reversal of hyperglycaemia (Vasir et al., 2000). MSCs from different sources have been shown to secrete a number of angiogenic factors that could potentially improve the survival of donor ECs and/or enhance the rate at which host ECs revascularise the transplanted islets. Despite this, there was no evidence of improved EC survival in the grafts of MSC co-transplanted mice or enhanced rate of islet revascularisation at three days, as shown through the very low CD34+ EC densities in both endocrine and non-endocrine components of the graft. ECs from different sized vessels, tissues and species show a reasonable degree of heterogeneity (Hewett and Murray, 1993, Cines et al., 1998). Taken together with the knowledge that the early post transplantation period is known to involve an active period of vascular remodelling, graft sections were stained with CD31 antibodies as an additional EC marker. Immunostaining of three day graft sections using CD31 antibodies showed that the density of CD31+ ECs in the stroma surrounding the islets in both transplant groups was far higher than that using CD34 as a microvascular EC marker. This taken together with low endocrine CD31+/CD34+ EC density, suggests that the majority of ECs previously observed in the endocrine tissue at one month after transplantation (see Figure 4.9 and Figure 4.10) are host derived, in support of other studies (Brissova et al., 2004, Nyqvist et al., 2005, Nyqvist et al., 2011). It is however acknowledged that it is difficult to fully determine the origin of ECs at the graft site, as we did not tag either donor or host ECs. Notably, a recent study that

utilised a Tie2-GFP mouse model, therefore specifically tagging ECs, showed that up to fifty percent of donor ECs actually migrated out of the islets into the surrounding tissue stroma (Nygqvist et al., 2011). The differential expression of EC markers within the graft non-endocrine component at three days after transplantation is indicative of the proliferative EC phenotype at this time point. This is supported by previous findings that CD34 antibodies do not bind to proliferating ECs (Fina et al., 1990), while CD31 is a pan-EC marker which has been shown to be expressed by proliferating ECs (Bencini et al., 1993).

A second experimental group was used to determine whether MSCs were able to improve islet graft revascularisation and function at seven days post transplantation. The two separate time points were chosen, as previous reports have shown that the revascularisation of transplanted islets begins 2-4 days after transplantation (Menger et al., 1989), concluding between 10-14 days post transplantation (Menger et al., 1992, Mendola et al., 1994, Merchant et al., 1997). The process is slower for islets functioning in a hyperglycaemic microenvironment (Sandler and Jansson, 1987, Jansson and Sandler, 1989, Vasir et al., 2000), moreover, hyperglycaemia can also induce apoptosis of islet ECs (Favaro et al., 2008). Thus, any affect of MSCs on glycaemia, may also correlate with secondary effects on vascular engraftment. Therefore, we wanted to investigate an intermediate time point, as well as the earlier three day time point, where any intervention would be more likely to improve donor EC survival than the revascularisation process itself. Unfortunately, it was not possible to correlate vascular engraftment with graft function at seven days, as the minimal islet mass model failed in this particular experiment, with better glycaemic control observed in control islet-alone mice than expected. The most likely reason for this is the lighter starting body weight of the mice transplanted with islets alone, compared to the previous one month study, where the minimal islet mass model worked well, with only 42% of control mice attaining normoglycaemia by one month. Therefore, future use of the minimal islet mass model should take into consideration the fact that 150 fresh islets is not a suitable number for mice of less than 22.5g body weight at the time of transplantation.

Experimental islet transplantation has until more recently, focused mainly on the renal capsule as an implantation site (Merani et al., 2008), largely due to the ease of retrieving the grafts for histological analysis. Therefore, many of the estimates for the time course of islet revascularisation are based on studies in which the islets have typically fused to form an amorphous mass of endocrine tissue within seven days post transplantation (Morini et al., 2007). Other studies have indicated that smaller islets revascularise better than large islets (Kampf et al., 2006) and it is therefore conceivable that reduced fusion between individual islets may correlate with an increased rate of vascular engraftment. Co-transplantation of MSCs did not produce such notable differences in graft morphology at seven days to those observed at one month post transplantation. However, differences in the extent of islet fusion were starting to emerge, with a 33 percent reduction in the average area of individual endocrine aggregates observed in the grafts of islet + MSC, compared to islet-alone recipients.

In accordance with the vascular engraftment studies at one month, extensive areas of islet-alone tissue were found to completely lack ECs at seven days. However, their number had increased relative to grafts analysed at three days, indicating that the revascularisation process was progressing, as expected (Miao et al., 2006, Nyqvist et al., 2011). The grafts of islet + MSC recipients were both quantitatively and structurally superior to those of islet-alone recipients, with ECs showing a more heterogeneous distribution throughout the islet tissue, with a greater vascular density observed. It is important to note that the vascular density in both transplant groups was not as high as that previously observed at one month (see Figure 4.9 and Figure 4.10), showing that the revascularisation process was not yet completed. The higher endocrine EC density of MSC co-transplanted mice implies that the islets co-transplanted with MSCs are already better oxygenated at seven days post transplantation, which is likely to enhance β -cell function (Dionne et al., 1993) and reduce hypoxia-related β -cell death (Davalli et al., 1996, Biarnes et al., 2002, Miao et al., 2006, Emamaullee et al., 2007). It is possible that the enhanced rate of islet revascularisation reflects an MSC-induced change in EC phenotype, which enables the ECs previously seen surrounding the graft at three days, to migrate into the islet tissue, possibly due to the secretion of matrix metalloproteases (Ding et al., 2009) and

subsequent degradation of the extracellular matrix (Ghajar et al., 2006, Potapova et al., 2007). In accordance, a recent *in vitro* study by Johansson and colleagues showed that MSCs can indeed enhance the ability of ECs to migrate into the islets, in a three-dimensional fibrin gel assay, in which human islets were coated with ECs either with or without MSCs (Johansson et al., 2008). The differential expression of CD34+ and CD31+ ECs in the non-endocrine tissue surrounding the islets in both transplant groups remained at seven days post transplantation, as well as the earlier three day time point. This is indicative that there are still a number of highly proliferative ECs at the graft site at seven days, as expected given that the EC density within the transplanted endocrine tissue has not yet reached the values previously observed at one month post transplantation where there were no longer signs of active remodelling, in either transplant group.

In summary, this study has provided additional insight into the time-course and processes involved in the remodelling of islets transplanted both alone and with MSCs beneath the kidney capsule; indicating that MSCs are able to increase the rate as well as the overall extent of islet revascularisation, thus potentially explaining the improved graft function seen in the early post transplantation period for the one month study (see Figure 4.3). Additional experiments should specifically address whether alterations in graft morphology, in the absence of MSCs or any additional ‘islet helper cell’, can also improve vascular engraftment and glycaemia in STZ-diabetic mice.

6 Chapter Six - Maintaining islet morphology for improved transplantation outcome

6.1 Introduction

Islets of Langerhans are small endocrine aggregates of limited size (50 – 500µm in diameter), dispersed throughout the exocrine pancreas, as opposed to being merged into a solid organ. The reasons for this are slightly obscure, but it is likely that there is some physiological relevance that ensures optimal islet function (Weir and Bonner-Weir, 1990). It is known that individual islets can synchronise their activity, which may provide greater efficiency of islet hormone secretion (Weir and Bonner-Weir, 1990). Additionally, this organisation allows the exocrine tissue to be supplied with islet hormones (Henderson, 1969).

Transplantation of islets as pellets at extrahepatic sites, such as the kidney subcapsular site, results in the fusion of individual islets and formation of large endocrine aggregates (Davalli et al., 1996, Biarnes et al., 2002). Oxygen tension gradients can develop (Carlsson et al., 1998) due to the pathological formation of these amorphous endocrine masses, which threatens the function (Dionne et al., 1993) and ultimately survival of transplanted islet cells (Davalli et al., 1996, Biarnes et al., 2002). During the enzymatic isolation process the islets lose not only their external vascular support, but also their peripheral basement membrane and extracellular matrix support (Wang and Rosenberg, 1999, Thomas et al., 1999, Thomas et al., 2001, Paraskevas et al., 2000). The loss of integrin signalling between islets and the surrounding extracellular matrix (ECM) proteins results in apoptosis – a process known as anoikis (Thomas et al., 1999, Frisch and Screaton, 2001, Pinkse et al., 2006). Therefore, it is crucial to restore islet-ECM interactions, as well as the islet vasculature during the early post transplantation period in order to minimise the substantial islet cell death that is known to occur (Davalli et al., 1996, Biarnes et al., 2002, Emamaullee et al., 2007).

Unfortunately, the microenvironment to which islets are transplanted clinically does not provide the islets with optimal trophic support, nor do many of the experimental

transplantation sites. Therefore, more efficient techniques for islet transplantation and engraftment are needed (Gibly et al., 2011, Carlsson, 2011). We have previously shown that co-transplantation of islets with mesenchymal stem cells (MSCs) produces profound alterations in graft morphology, maintaining normal islet size and architecture. Notably, this was also associated with increased vascularisation of the transplanted endocrine tissue and better glycaemic control in diabetic recipients (see Chapter Four). In addition, analysis of the graft non-endocrine tissue showed that there were extensive areas surrounding the transplanted islets consisting of collagen fibres. As well as the MSC-dependent effects on graft morphology, it is well established that MSCs secrete a number of trophic soluble mediators, which are likely to improve islet function and/or revascularisation. Therefore, the aims of the current study were to determine whether maintaining islet organisation and morphology in the absence of an additional ‘islet helper cell’, such as MSCs, would also improve transplantation outcome.

6.2 Methods

6.2.1 Transplantation of pelleted and dispersed islets

Recipients of pelleted islets were transplanted in the conventional way as described in chapter two. Briefly, groups of 150 fresh islets were suspended in media and centrifuged/pelleted immediately before transplantation, into PE50 polyethylene tubing. The pelleted islets were then implanted as a single cluster of cells and are referred to as the ‘pelleted islets’ transplant group. For mice transplanted with islets that were spread out under the kidney capsule; in contrast to islets transplanted in the conventional way as a single cluster, islets were not centrifuged/pelleted into the PE50 polyethylene tubing before transplantation. Groups of 150 fresh islets were suspended in media and were aspirated into the PE50 polyethylene tubing immediately prior to transplantation, ensuring that they were not aggregated together. Islets were implanted underneath the kidney capsule in a similar way using a Hamilton syringe and PE50 polyethylene tubing, but instead of implanting the islets as a single aggregate, the islets were spread out beneath the kidney capsule, over the majority of the upper surface of the kidney. Mice transplanted in this way are referred to as the ‘dispersed islet transplant’ group.

6.2.2 Transplantation of islets in matrigel plugs

Matrigel (BD marathon growth factor reduced) was kept at -20 until use. 250µl aliquots were defrosted at 4°C overnight before transplantation. Sterile PBS, 1.5ml eppendorfs tubes, 50ml falcon tubes, 1ml syringes, 23 gauge needles, 1000µl, 200µl and 20µl pipette tips were also cooled at 4°C overnight. Heparin and PBS were gently added to matrigel aliquots using a pipette and mixing, whilst avoiding the formation of air bubbles, as below:

Preparation of matrigel solution

For each 250µl aliquot of defrosted matrigel

<u>Reagent</u>	<u>Volume (µl)</u>
Heparin (40U/ml)	22.4
Matrigel	250
PBS	72.6

A total volume of 400µl of matrigel solution was used per mouse. Each reagent was added carefully with sterile plasticware taking care not to introduce bubbles into the solution.

150 fresh islets were mixed with 50µl of matrigel solution and the remaining solution was needed to fill the dead space in the Hamilton syringe at the time of transplantation. The matrigel solution/islets and all materials were kept on ice until transplantation to maintain the fluidity of the matrigel.

6.3 Results

6.3.1 Efficacy of pelleted and dispersed islet transplants *in vivo*

Dispersion of the islet transplant underneath the kidney capsule produced superior transplantation outcomes compared to that of islets transplanted as a single pellet/cluster of islets, as shown in Figure 6.1. The average blood glucose concentrations of mice with dispersed islet transplants was significantly lower than that of mice transplanted with pelleted islets at 21 and 28 days post transplantation (Figure 6.1a). After one month, only 17% of mice transplanted with pelleted islets had cured, compared to 83% of mice with dispersed islet transplants (Figure 6.1b). The

average time to reverse hyperglycaemia for mice with dispersed transplants was 23.8 ± 1.7 days, with only one mouse in the pelleted islets transplant group curing at all, at 28 days post transplantation. There were no significant differences in the weights of mice in either transplant group on day 0 (21.0 ± 0.5 and 22.6 ± 0.4 g, for mice with pelleted islet and dispersed islet transplantations respectively, $p = 0.8$, Student's t test) or at one month post transplantation ($22.8.0 \pm 1.0$ and 24.4 ± 0.5 g, $p = 0.3$, Student's t test). All nephrectomised mice reverted to hyperglycaemia within two days. Figure 6.1c shows the average blood glucose concentrations of mice in the previous MSC co-transplantation study to allow for comparison between this study and the current study. In contrast to the MSC co-transplanted mice, where glycaemia was improved relative to control islet-alone recipients (equivalent to the pelleted islet group in the current study) from day three after transplantation and throughout the one month study; dispersion of islets beneath the kidney capsule did not have any significant effect on glycaemia until 21 days post transplantation Figure 6.1d.

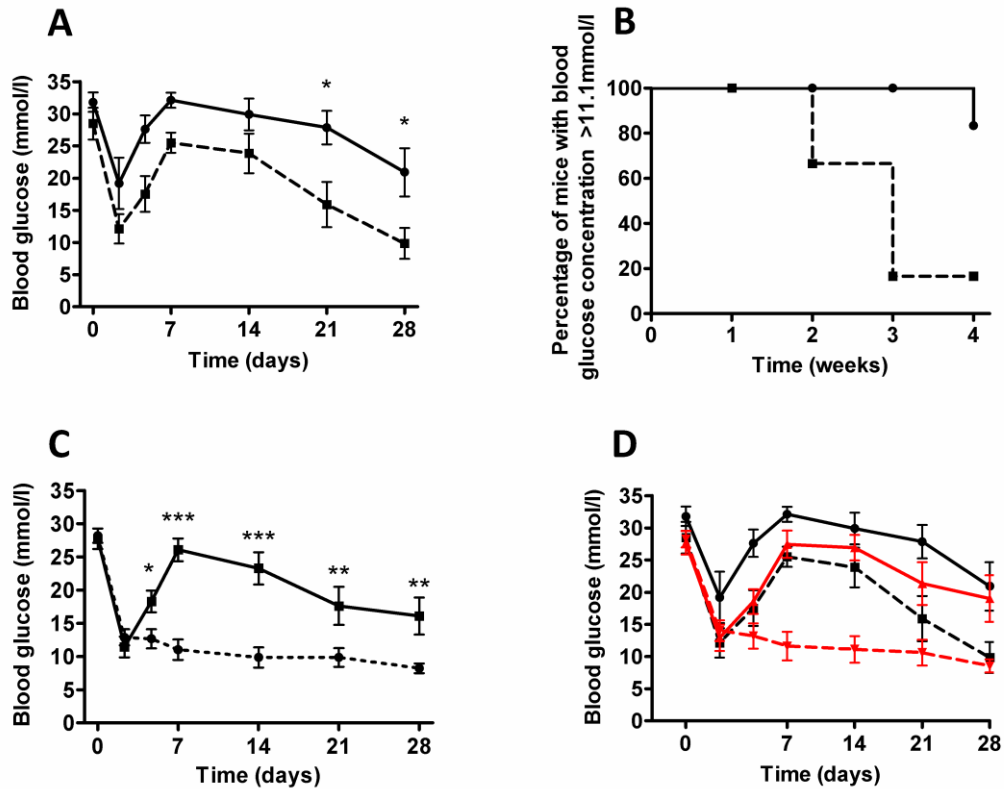


Figure 6.1 Efficacy of pelleted and dispersed islets *in vivo*. **A.** Blood glucose concentrations of mice with pelleted (continuous line) or dispersed islet transplantation (dashed line), * $p < 0.05$, Two-Way RM ANOVA with Bonferroni post hoc test, $n=6$ for both transplant groups. **B.** Percentage of mice remaining diabetic (blood glucose concentration >11.1 mmol/l) after transplantation as in A, $p = 0.02$ Kaplan–Meier, $n=6$ for both transplant groups. **C.** Blood glucose concentrations of mice transplanted with 150 islets alone (continuous line) or those co-transplanted with 150 islets + 25×10^4 kidney-derived MSCs (dashed line) as shown previously in chapter four, figure one; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs mice transplanted with islets + MSCs (RM ANOVA with Bonferroni post hoc test, $n=13$). **D.** Blood glucose concentrations of mice transplanted with dispersed transplants (black dashed line), pelleted islets (black continuous line), islet-alone transplanted mice from one month MSC-co transplantation study (red continuous line) and islet + MSC co-transplanted mice from one month MSC-co transplantation study (red dashed line).

6.3.2 Morphology of pelleted and dispersed islet grafts

Figure 6.2 shows the morphology of graft material retrieved at one month post transplantation, demonstrating that the technical procedure of dispersing islets beneath the kidney capsule was able to maintain the typical size and morphology of endogenous pancreatic islets to some extent, in comparison with the amorphous mass of endocrine tissue formed in the control pelleted islets transplant group. As previously demonstrated for islets transplanted alone and as a single pellet (see Figure 4.5), insulin immunostaining of the graft sections from mice transplanted with

pelleted islets revealed a single amorphous mass of aggregated insulin-positive endocrine tissue in the majority of sections (Figure 6.2a). In contrast, for most of the graft sections from dispersed islet transplant recipients, there was little evidence of any fusion between individual islets, with multiple small endocrine aggregates clearly discernable (Figure 6.2b). The morphology of the dispersed islet grafts was however more variable than that of control mice. In a small number of sections analysed (from one mouse only) there were signs of islet fusion with large endocrine aggregates present. The extent of islet fusion was quantified using the system described previously in detail (see Chapter Four), to determine the extent to which manually spreading islets at the implantation site can prevent the formation of large aggregated endocrine masses. The average area of each single endocrine aggregate per graft section in the dispersed islet grafts was approximately half of that seen for pelleted islet grafts (Figure 6.2c). There was also a trend towards the total endocrine area per graft section being higher for mice transplanted with pelleted islets, compared with those transplanted with dispersed islets ($91,377 \pm 5,047$ and $41,411 \pm 16,128 \mu\text{m}^2$, $n=3-5$, $p = 0.06$). The total graft area per section (endocrine + non-endocrine tissue) was approximately 50 percent higher in the pelleted islets group compared to the dispersed islets, although this was not significant ($115,298 \pm 8728$ and $66,764 \pm 18931 \mu\text{m}^2$, pelleted islets and dispersed islets respectively, $p = 0.11$, $n=3-5$). The graft composition was similar, in terms of the percentage of graft material occupied by endocrine tissue (79.6 ± 0.6 and $66.7 \pm 8.1\%$ endocrine tissue (staining positively with insulin antibodies) for pelleted and dispersed islet grafts, $n=3-5$, $p = 0.2$), thus the higher total graft area per section in the pelleted islet grafts, was not due to the presence of additional non-endocrine stromal tissue.

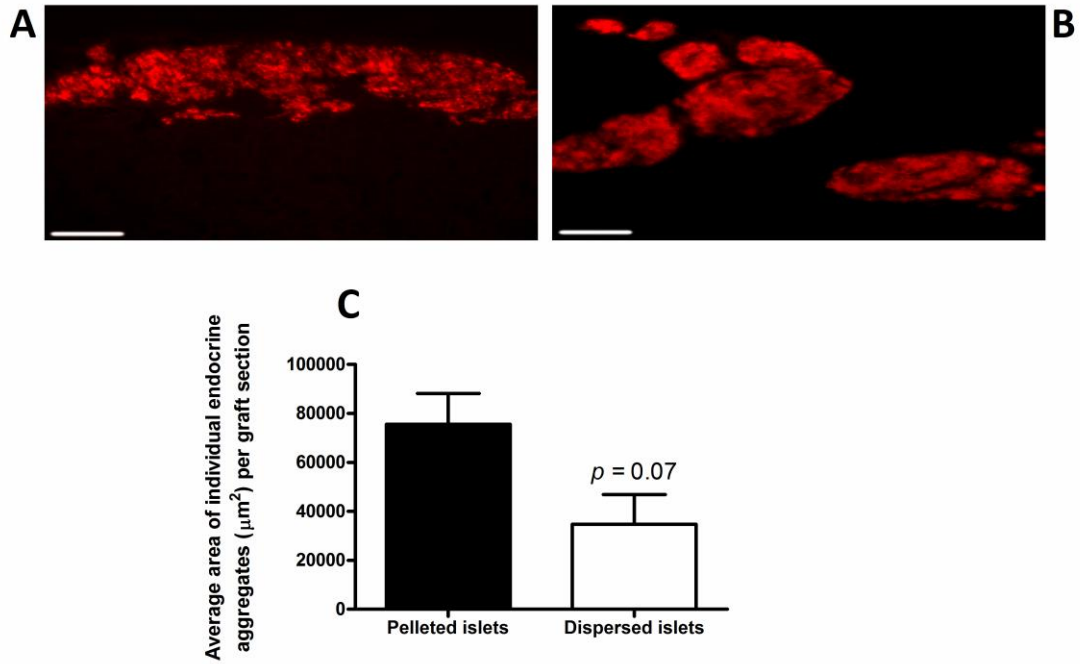


Figure 6.2 Morphology of pelleted and dispersed islet grafts. Representative sections of pelleted islet (a) and dispersed islet grafts (b) at one month post transplantation. **A.** Pelleted islet graft, where islets have typically aggregated to form a single amorphous endocrine mass. **B.** Dispersed islet graft, where individual islets are still clearly discernable, original magnification $\times 100$, scale bars are $100\mu\text{m}$. **C.** Average individual endocrine aggregate area in graft sections; $n = 3-5$ animals per group, $p = 0.07$, Student's t test.

6.3.3 Composition of pelleted and dispersed islet grafts

Immunostaining of graft sections with glucagon antibodies showed that the islet architecture in control mice transplanted with pelleted islets was disorganised (Figure 6.3a), as previously shown for islet-alone recipients at one month post transplantation (Figure 4.7). For mice with dispersed islet grafts there was variation between mice, but the peripheral rim of α -cells was clearly evident in many of the graft sections; particularly where individual islets could still be clearly discerned (Figure 6.3b). The percentage of α -cells was similar between transplant groups (Figure 6.3c).

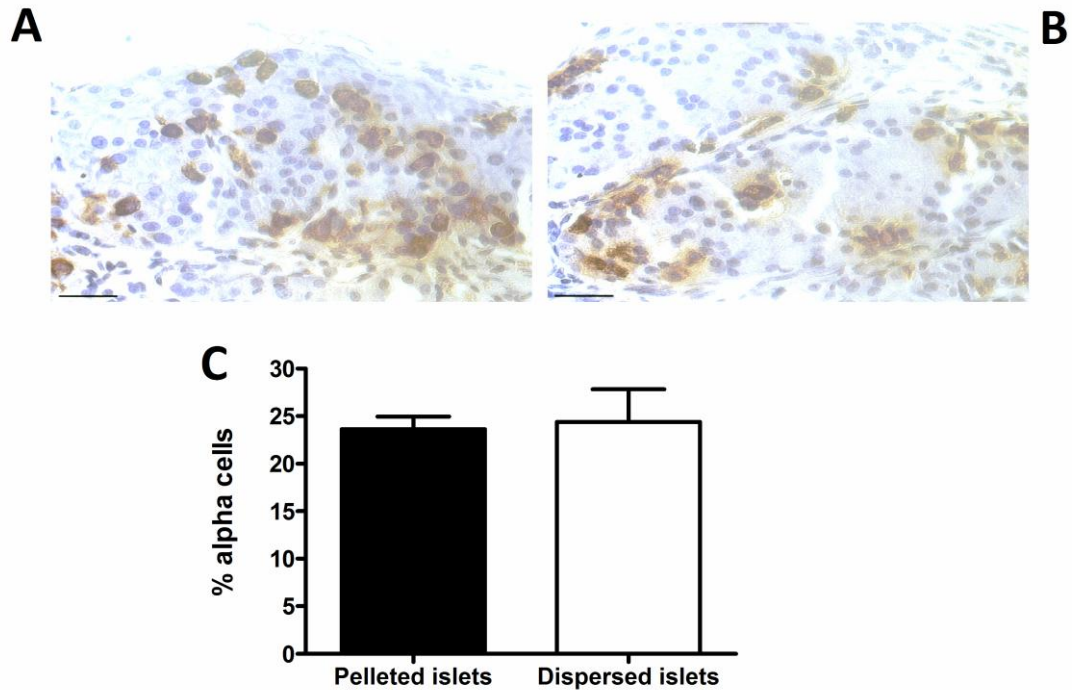


Figure 6.3 Composition of pelleted and dispersed islet grafts. Distribution of glucagon-positive α -cells. At one month post transplantation sections were stained with glucagon antibodies in grafts consisting of pelleted islets (a) and dispersed islets (b). Images are representative of sections from 3-5 animals in each transplant group, original magnification $\times 400$, scale bars are 25 μ m. C. The percentage of α -cells in the same graft sections was quantified, $n=3-5$, $p = 0.85$, Student's t test.

6.3.4 Vascular density of pelleted and dispersed islet grafts

Immunostaining of islet grafts with CD34 antibodies indicated that there were areas of endocrine tissue devoid of ECs in the pelleted islet grafts (Figure 6.4a), as previously shown at one month post transplantation, for islets transplanted alone using the same transplantation method. There was a more homogeneous distribution of ECs throughout the smaller endocrine aggregates present in the graft sections of mice with dispersed islet grafts, with smaller areas of endocrine tissue devoid of ECs (Figure 6.4b). The number of ECs in the endocrine and non-endocrine tissue was quantified. The mean endocrine vascular density was 60 percent higher in the grafts consisting of dispersed islets compared to those consisting of pelleted islets, but this was not statistically significant, as shown in Figure 6.4c. There were no significant differences in the vascular density of the non-endocrine tissue (Figure 6.4c). The average area of individual endocrine aggregates per graft section was correlated with the mean

endocrine vascular density, with a trend towards a correlation showing that EC density decreases as the average area of endocrine aggregates increases (Figure 6.4d).

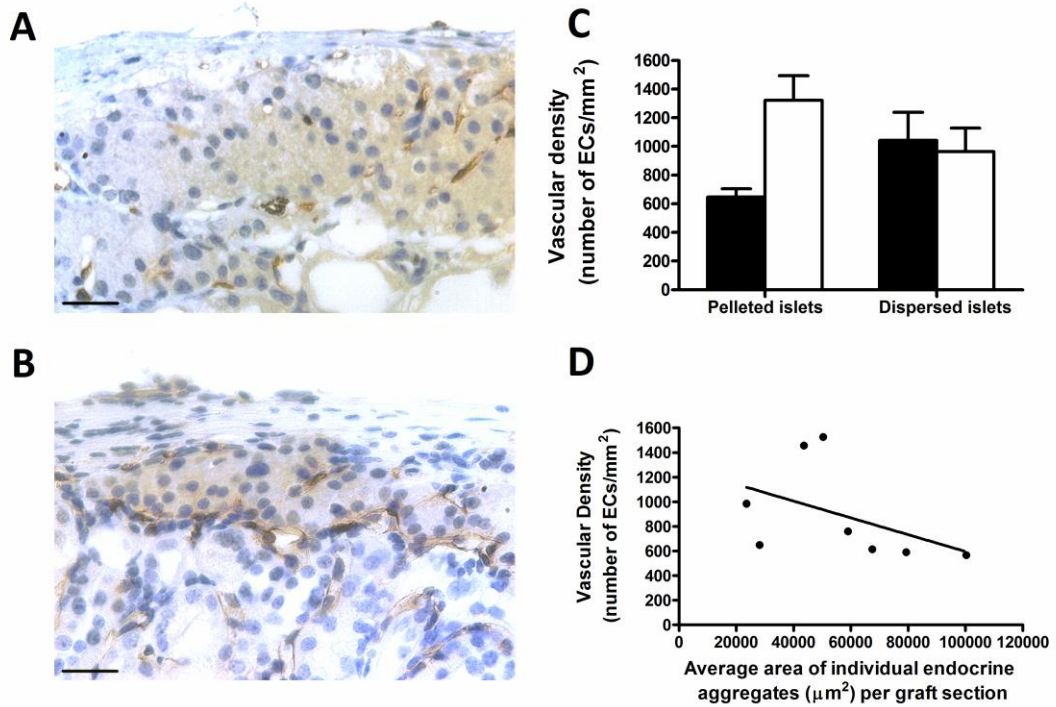


Figure 6.4 Vascular density. Staining of microvascular ECs with CD34 antibodies in grafts consisting of pelleted islets (a) and dispersed islets (b). Images are representative of sections from 3-5 animals in each transplant group, original magnification x400, scale bars are 25 μm . **C.** Vascular density of endocrine components (black bars) and non-endocrine components (white bars) in one month grafts consisting of pelleted islets or dispersed islets, $n=3-5$, $p > 0.05$, Two-Way ANOVA with Bonferroni post hoc test. **D.** Correlation between average area of individual endocrine aggregates and endocrine vascular density for grafts in both transplant groups, $p = 0.06$, Spearman's rank correlation.

6.3.5 Matrigel implants in mice at time of transplantation

A second approach was used to prevent the fusion of islets beneath the kidney capsule, thereby inducing a morphology that more closely resembles that of islets in the endogenous pancreas. For this strategy; islets were transplanted beneath the kidney capsule in the conventional way as a single pellet, or spread out in matrigel and implanted beneath the kidney capsule. Visualisation of the graft-bearing kidneys under a dissecting microscope at the time of transplantation revealed distinct differences in the appearance of the grafts, with islets clearly present as a single pellet in control mice (Figure 6.5a). In contrast, islets transplanted in matrigel occupied a greater area underneath the kidney capsule, with fewer signs of individual islets aggregating together (Figure 6.5b).

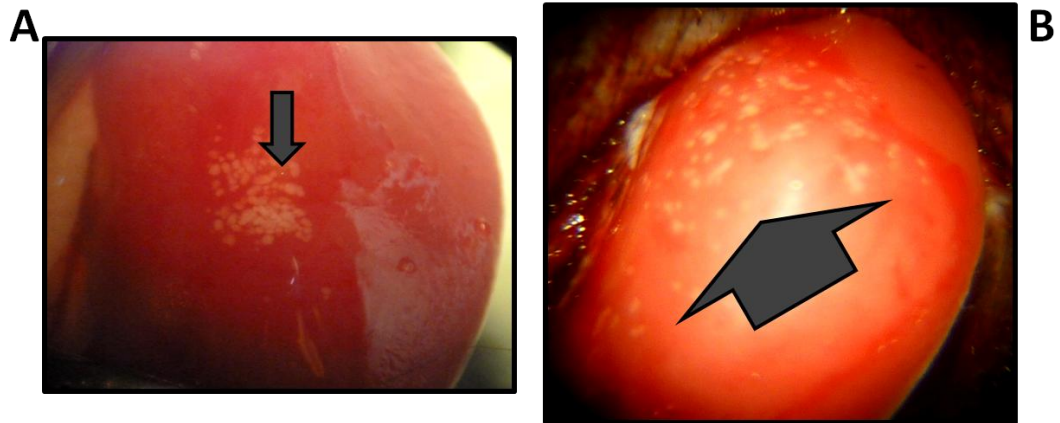


Figure 6.5 Photographs of islet-graft bearing kidneys immediately after transplantation. Islets transplanted in the conventional way as a single islet pellet (a) and islets spread underneath the kidney capsule in matrigel (b). Photographs were taken with a digital camera (Panasonic Lumix DMC-ZX1) through a dissecting microscope. Arrows indicate the location of the islet graft.

6.3.6 Matrigel implants in mice at one month post transplantation

At one month post transplantation the graft-bearing kidneys were harvested and visualised under a dissecting microscope. The implanted islets were identifiable in both transplant groups, but their appearance was clearly different, as shown in Figure 6.6. The grafts of mice transplanted with pelleted islets were present as a single mass of compacted islets (Figure 6.6a). The grafts of mice transplanted with dispersed islets appeared to maintain the same morphology as was present on the day of transplantation, with islets dispersed over a larger area beneath the kidney capsule (Figure 6.6b).

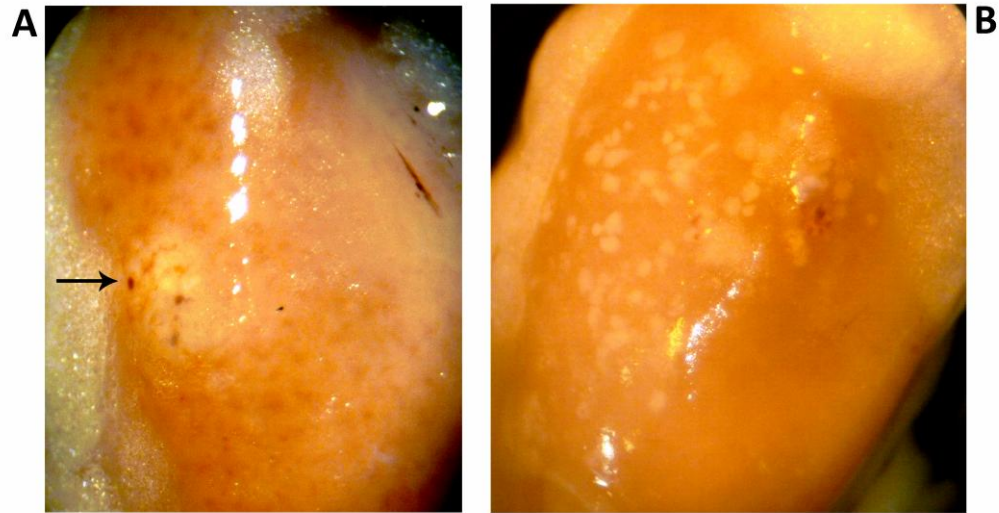


Figure 6.6 Photographs of islet-graft bearing kidneys at one month post transplantation. Islets transplanted in the conventional way as a single islet pellet (**a**) and islets spread beneath the kidney capsule in matrigel (**b**), at one month after transplantation. Photographs were taken with a digital camera (Panasonic Lumix DMC-ZX1) through a dissecting microscope. Arrow indicates aggregated islets.

6.3.7 Efficacy of pelleted islets and islets dispersed in matrigel

Transplantation of islets spread out in matrigel plugs produced superior transplantation outcomes to that of pelleted islets. The average blood glucose concentrations of islet-matrigel implanted mice were significantly lower than in mice transplanted with pelleted islets at 1 and 14 days post transplantation (Figure 6.7a). The curative capacity of islets in matrigel was better than that of pelleted islets, with 3/3 islet-matrigel implanted mice curing by one month, compared to only 1/4 control mice transplanted with pelleted islets (Figure 6.7b). There were no significant differences in the starting weight of mice in either transplant group on day 0 (26.2 ± 1.8 and 25.5 ± 0.4 g, pelleted islets alone and islet-matrigel implanted recipients respectively, $n=3-4$, $p = 0.8$) or at one month post transplantation (27.1 ± 2.2 and 28.2 ± 0.5 g, $n=3-4$, $p = 0.7$).

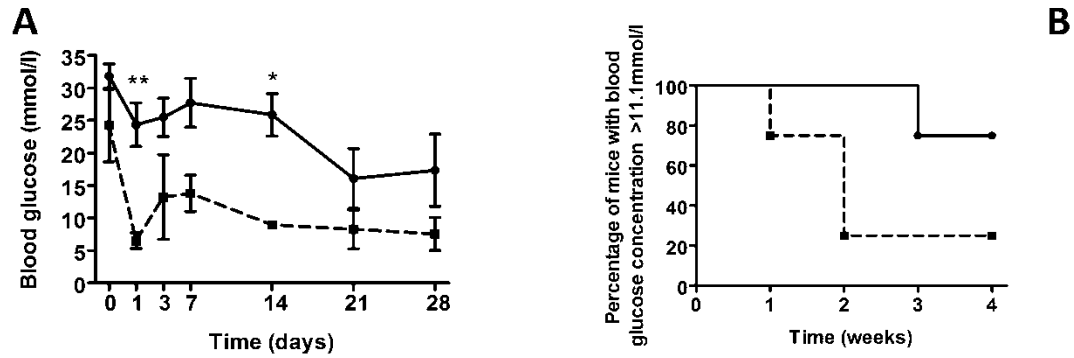


Figure 6.7 Graft function **A.** Blood glucose concentrations of mice transplanted with pelleted islets (continuous line) or islets in matrigel (dashed line), beneath the kidney capsule, $*p < 0.05$, $**p < 0.01$, Two-Way RM ANOVA with Bonferroni post hoc test, $n=3-4$. **B.** Percentage of mice remaining diabetic (blood glucose concentration >11.1 mmol/l) after transplantation as in A, $p = 0.02$ Kaplan–Meier, $n= 3-4$.

6.4 Discussion

The function and survival of transplanted islets is compromised by a number of problems associated with the microenvironment to which they are transplanted. In particular, islets transplanted beneath the kidney capsule are known to fuse with each other, which has been recognised as a pathological feature of morphological engraftment (Davalli et al., 1996, Biarnes et al., 2002). In the current study, we have used two approaches to essentially prevent this fusion process, which results in the formation of large amorphous endocrine aggregates. The first approach was to alter the technical procedure used to transplant islets beneath the kidney capsule; manually spreading the islets under the kidney capsule, as opposed to transplanting them as a single pellet or cluster. In the second approach, islets were mixed with and transplanted in a relatively large volume of matrigel (considering the space beneath the kidney capsule), which forms a solid plug at 37°C ensuring that the islets spread out beneath the kidney capsule.

The current study supports the suggestion that islet fusion is detrimental for transplantation outcome (Davalli et al., 1996, Biarnes et al., 2002). The morphological studies showed that dispersing islets beneath the kidney capsule reduces the size of individual endocrine aggregates by approximately half compared to grafts consisting of pelleted islets. Importantly, in contrast to the previous MSC co-transplantation

study, there was little evidence of significant amounts of non-endocrine stromal tissue surrounding the dispersed islets, as is evident from the finding that the total graft area was also decreased by approximately 50 percent in the dispersed islet transplant group. Thus, the positive effects of dispersing the islet graft are purely due to a reduction in the area of individual endocrine aggregates, as opposed to any beneficial trophic effect of collagen or other ECM proteins (Jalili et al., 2011). The formation of smaller endocrine aggregates at the implantation site may well account for the better glycaemia after 21 days in mice receiving dispersed islet transplants compared to those transplanted with pelleted islets; simply because the magnitude of the oxygen gradient is reduced, thereby providing more viable functional tissue. Indeed, this has been suggested by O'Sullivan and colleagues, who showed that encapsulated small islet aggregates were superior to larger intact islets, in terms of *in vitro* function and curative capacity when transplanted to diabetic mice, which was associated with reduced hypoxia-related necrosis of islet tissue (O'Sullivan et al., 2010). Importantly, the O'Sullivan study utilised encapsulated islets or islet aggregates, which are unable to revascularise *in vivo*, as non-encapsulated islets do. Therefore, the improved islet function was independent of any influence on islet revascularisation. In support of the hypothesis that large endocrine aggregates may be detrimental to islet function and survival due to hypoxia-related islet dysfunction (Dionne et al., 1993) and cell death (Davalli et al., 1996, Biarnes et al., 2002, Miao et al., 2006, Emamaullee et al., 2007), Carlsson and colleagues demonstrated the existence of oxygen tension gradients throughout fused endocrine tissue, with a trend towards a higher partial pressure of oxygen (pO_2) at the periphery of the aggregated islet graft tissue, compared with the centrally located parts of the graft (Carlsson et al., 1998).

The results of the current study are in accordance with findings related to the variable success rates of intramuscular islet transplantation. Interestingly, Christoffersson and co-authors found that islets transplanted intramuscularly as clusters were subject to the development of fibrosis at central parts of the graft (Christoffersson et al., 2010), in comparison to islets transplanted in a 'pearls-on-a-string' fashion, which ensures that they are engrafted as single islets and is associated with more favourable transplantation outcomes (Lund et al., 2010). This is important as it suggests that the beneficial impact of spreading islets at the implantation site is not site-specific.

Intriguingly, a similar situation appears to occur during the pre-transplant period. The Edmonton group have previously reported that compaction of islets through prolonged periods of maintaining the islets in a pelleted state, prior to transplantation, is detrimental for transplantation outcome in terms of graft function. This was associated with increased hypoxia-related β -cell apoptosis in the islets which had been maintained in a pelleted state for 30 minutes before transplantation, compared to those which were suspended in media and only pelleted immediately before transplantation (Merani et al., 2006). Thus, important alterations to the technical aspects of islet isolation, purification and transplantation may help to reduce ischemia-related islet cell injury and death, thereby providing more efficient utilisation of islet tissue, which is currently in short supply.

Revascularisation is a complex multi-step process involving the digestion of the vascular wall by proteases and the migration, proliferation and differentiation of ECs (Conway et al., 2001). A number of reports have demonstrated inadequate revascularisation of transplanted islets at a number of implantation sites (Mattsson et al., 2002b, Mattsson et al., 2003, Olsson and Carlsson, 2005, Lau and Carlsson, 2009). This is at least partially attributed to the difficulties associated with ECs from the host implantation organ being able to physically degrade the islet ECM and migrate into the islet tissue. Interestingly in this context, Kampf and co-authors demonstrated the superior vascular engraftment of small, compared to larger islets (Kampf et al., 2006). Similarly, our previous study also demonstrated that the smaller endocrine aggregates present in the grafts of MSC co-transplanted mice were better vascularised than the large endocrine masses formed in mice implanted with islets alone (the islets were maintained in suspension and pelleted prior to transplantation for both groups). Thus, it might be expected that the histological graft sections from mice with dispersed islet grafts in the current study, would have an enhanced endocrine EC density compared to control mice transplanted with pelleted islets. Although the endocrine EC density was 60 percent higher in the grafts consisting of dispersed islets compared to pelleted islets, this was not statistically significant, reflecting the large variation in graft morphology and vascularisation, observed in the dispersed islet transplant group. There was a trend towards a correlation between the average individual endocrine aggregate area and endocrine vascular density, with

smaller endocrine aggregates being better vascularised than the larger aggregates. This at least partially supports our previous suggestion that MSCs may enhance islet graft revascularisation through MSC-dependent alterations in graft morphology.

A limitation of spreading islets beneath the kidney capsule as a method for preventing islet fusion is that this technique is difficult to consistently reproduce similar graft morphology in all recipients. Therefore, we used a second strategy to prevent the formation of large endocrine aggregates at the graft site. This involved the use of matrigel plugs of a defined volume, which should ensure that islets are dispersed to a similar extent in each animal, assuming that the islets are mixed in with the matrigel in the same way. Our previous MSC co-transplantation study showed that there were extensive areas of non-endocrine tissue consisting of loosely packed collagen fibres surrounding the MSC co-transplanted islets, in contrast to control islet-alone recipients where the majority of graft tissue consisted of endocrine cells with little evidence of significant amounts of supportive connective tissue stroma. It seems likely that the MSC-induced alterations in graft morphology produced beneficial effects on islet function through the maintenance of islet organisation and morphology, but also through the provision of additional ECM trophic support. Thus, implantation of islets in matrigel plugs may provide a better strategy for mimicking the microenvironment provided by MSC co-transplantation than technically dispersing the islets beneath the kidney capsule.

Matrigel is a solubilised basement membrane preparation extracted from an Engelbreth-Holm-Swarm mouse sarcoma (Swarm, 1963), which is liquid when cold, but solidifies/gels at room temperature, thereby forming 'plugs' when injected beneath the kidney capsule. It has previously been demonstrated that subcutaneous transplantation of islets in matrigel plugs can successfully reverse hyperglycaemia in diabetic mice (Bharat et al., 2005), indicating that matrigel does not exert any detrimental effects on islet function. Furthermore, a number of reports have indicated that entrapment of islets within ECM scaffolds can enhance islet function (Perfetti et al., 1996, Nagata et al., 2001, Kaido et al., 2006, Jalili et al., 2011). During the enzymatic isolation process the islets lose not only their external vascular support, but also their peripheral basement membrane and ECM support (Thomas et al., 1999,

Paraskevas et al., 2000, Pinkse et al., 2006), with a number of studies demonstrating that the lack of native ECM components contribute to substantial islet cell death during the immediate post transplantation period (Rosenberg et al., 1999, Thomas et al., 1999). It has also been demonstrated that ECM components have the capacity to enhance β -cell survival (Hammar et al., 2004, Pinkse et al., 2006, Perfetti et al., 1996, Zhao et al., 2010), differentiation (Jiang and Harrison, 2002) and insulin secretion (Bosco et al., 2000). The main components of matrigel are ECM proteins, such as laminin, collagen IV, fibronectin and perlecan (Hughes et al., 2010). Given that collagen IV, laminin and fibronectin are the major basement membrane proteins present between intraislet ECs and endocrine cells (van Deijnen et al., 1992, Van Deijnen et al., 1994, Pinkse et al., 2006), it could therefore be expected that implantation of islets in matrigel might help to restore islet-ECM interactions more rapidly and efficiently than when implanting islets alone; thereby minimising the substantial islet cell death associated with the lack of native ECM components at the implantation site (Rosenberg et al., 1999, Thomas et al., 1999). Indeed, a recent study by Jalili and colleagues showed that collagen matrix improves islet survival and function *in vitro*, in comparison to islets cultured using a regular two-dimensional culture system. Additionally it was shown that transplantation of islets in the same collagen matrix reduced the number of islets required to achieve normoglycaemia in diabetic mice (Jalili et al., 2011), confirming another study which showed that adsorption of collagen and other ECM proteins to hydrolyzed-scaffolds had a positive effect on transplantation outcome (Salvay et al., 2008). In accordance with these recent reports, the present study shows that implantation of islets in matrigel plugs improved glycaemic control in diabetic mice at 1 and 14 days after transplantation, suggesting that matrigel may provide a more suitable microenvironment to that where islets are transplanted alone beneath the renal capsule. Indeed, Blomeier et al have emphasised the benefits of using biomaterials or scaffolds as a method to provide spatial and functional support for the islets (Blomeier et al., 2006).

Visualisation of the grafts under a dissecting microscope, on both the day of transplantation and one month after transplantation showed that the islet graft occupied a far larger area underneath the kidney capsule for islet-matrigel implanted mice compared to pelleted islet recipients. Moreover, there did not appear to be any

significant differences in the appearance of the matrigel-islet implants on the day of transplantation and one month later when the graft-bearing kidneys were harvested, indicating that there was no significant fusion of islets during the engraftment process. Histological analysis will provide a more detailed evaluation of graft morphology, however these preliminary findings strongly suggest that implantation of islets using matrigel plugs appears to provide a suitable method for ensuring the dispersion of islet grafts beneath the kidney capsule.

Implanting islets in matrigel plugs had no effect on glycaemia during the early post transplantation period, nor did dispersing islets beneath the kidney capsule. Given that MSC co-transplantation improved average blood glucose concentrations already at three days after transplantation; this suggests that MSCs do have additional functional properties that are independent of their effect on morphological and vascular engraftment. In this context, matrigel could potentially provide a scaffold for the entrapment of MSCs within the matrigel plug, dispersed amongst the islets, thereby maximising the potential for positive paracrine effects of MSCs. Combining the use of MSCs and ECM scaffolds may well help to enhance glycaemic control during the initial days post transplantation, due to an array of positive effects that MSCs have been shown to exert upon vascular engraftment, islet survival and function, as well as the host's immune system.

In the present study we deliberately chose to use growth factor reduced matrigel, as we wanted to reduce the influence of the high concentrations of angiogenic factors present in the growth factor supplemented matrigel. Indeed, previous studies have shown that optimal blood vessel formation in growth factor reduced matrigel transplanted subcutaneously required supplementation with both VEGF and HGF (Golocheikine et al., 2010). Thus, the revascularisation of islets implanted in matrigel appears to be suboptimal in the absence of any additional modulation. However, it is important to consider the influence of implantation site, as Golocheikine and colleagues used the subcutaneous site, which is known to be less well vascularised than the renal subcapsular site. The superior graft function observed in the current study with matrigel implants may well be related to an increased rate or extent of islet revascularisation, compared to pelleted islet recipients. Histological analysis of graft

morphology and vascularisation should provide interesting insight into potential mechanisms through which matrigel is affecting graft function. Renal subcapsular islet-matrigel implants are likely to provide a good model for studying factors that may influence the revascularisation process. Thus future studies could potentially determine the relative importance of maintaining islet organisation and morphology compared to that of the presence of ECM proteins, by transplanting pelleted or dispersed islets in matrigel plugs and assessing the impact this has on graft function and vascular engraftment.

It is acknowledged that additional experiments are needed to fully determine the influence of transplanting islets in matrigel plugs upon graft function, as well as morphological and vascular engraftment; however these preliminary findings indicate that matrigel does appear to have a positive influence on transplantation outcome. Moreover, the present study provides important information regarding the previous MSC co-transplantation study. In particular, it suggests that MSCs have additional functional properties to their observed positive effect on islet graft remodelling, which are likely to contribute to the improvements in glycaemia seen during the initial days post transplantation in the previous one month co-transplantation study (see Chapter Four). Therefore, subsequent experiments in this thesis have investigated the influence of MSCs directly on islet function *in vitro*, where any effect of MSCs must be independent of any influence on morphological and vascular engraftment.

In summary, the two approaches used to maintain normal islet organisation and morphology both showed successful outcomes in terms of preventing the islet fusion which typically occurs when islets are implanted beneath the kidney capsule. Moreover, both strategies produced superior transplantation outcome in terms of glycaemic control in recipient animals, supporting our previous observations that the maintenance of normal islet size and morphology is important for graft function. These studies suggest that any strategies to improve islet engraftment and function through changing the implantation site, should take into consideration the detrimental impact that transplanting islets as clusters can have. Preventing the fusion of individual islets at the implantation site is likely to provide a more suitable microenvironment for transplanted islets, ensuring greater utilisation of donor islet

tissue, which is essential for enabling the more widespread application of islet transplantation as a treatment for T1DM.

7 Chapter seven - Preculture of islets with MSCs for improved transplantation outcome

7.1 Introduction

The development of the Edmonton protocol resulted in dramatically improved clinical islet transplantation success (Shapiro et al., 2000). Although some centres have had successful outcomes with single-donor transplantations (Hering et al., 2004, Posselt et al., 2010, Matsumoto, 2011), the large majority of islet transplant recipients require multiple donors (Montaña et al., 1993, Shapiro et al., 2000, Ryan et al., 2005a). Given the current shortage of donor islet material, this means that islet transplantation is currently restricted to only a small subset of patients with severe hypoglycaemia unawareness, glycaemic lability or simultaneous with or after kidney transplantation. Efforts to reduce the number of donors required for each patient are therefore clearly warranted.

The success of the Edmonton protocol was partially attributed the use of freshly isolated, as opposed to cultured islets. However, there has been a return to the use of cultured islets for clinical transplantations, largely because of logistical reasons (Goss et al., 2002, Shapiro et al., 2003, Hering et al., 2004, Froud et al., 2005, Kin et al., 2008). In the clinical situation, culturing islets is beneficial as it provides a window of opportunity for quality control testing, time for shipment of islets to transplant centres (Weir and Bonner-Weir, 1997) and time for the initiation of time-dependent immunosuppressive protocols (Kin et al., 2008).

There are also additional benefits of islet culture in terms of β -cell function and survival following transplantation. Cultured islet preparations have improved immunogenicity (Kedinger et al., 1977) and reduced contaminating exocrine tissue, which is beneficial for transplantation outcome, as the acinar tissue releases harmful digestive enzymes (Kin et al., 2008), which are detrimental to β -cell survival during the immediate post transplantation period. Furthermore, low purity of islet

preparations is also associated with reduced revascularisation (Heuser et al., 2000), which can hamper β -cell function and survival in the longer term.

Despite the numerous benefits of islet culture, it is clear that there are also a number of disadvantages. Chapter three demonstrated the loss of both β -cells and endothelial cells (ECs) during short term culture, supporting a number of other studies showing impaired EC survival (Parr et al., 1980, Nyqvist et al., 2005, Olsson et al., 2006) and reduced functional β -cell mass for both human and rodent islets (Wang and Rosenberg, 1999, Keymeulen et al., 2006, Ichii et al., 2007, Kin et al., 2008). The loss of islet ECs during pre-transplantation tissue culture is a concern because experimental studies suggest that neovascularisation of transplanted islets involves both host and donor ECs (Vajkoczy et al., 1995, Linn et al., 2003b, Brissova et al., 2004, Nyqvist et al., 2005, Nyqvist et al., 2011). The loss of islet ECs before transplantation may therefore decrease the rate of revascularisation of the graft and contribute to the loss of islet function in the immediate post-transplantation period. Strategies to maintain endocrine and EC cell function prior to transplantation would clearly help to reduce the number of donors needed for each transplant recipient, thereby making better use of the donor islet material that is available.

Chapter Four demonstrated that mesenchymal stem cell (MSC) co-transplantation improves islet graft function in diabetic mice, which was correlated with alterations in graft morphology and enhanced vascular engraftment *in vivo*. In addition to their properties *in vivo*, MSCs also have a number of functional properties making them ideal cellular candidates for improving islet quality during the pre-transplant period. Numerous studies have shown that MSCs secrete an array of trophic factors (Park et al., 2009, Sordi et al., 2010, Xu et al., 2008, Karaoz et al., 2010), that are likely to favour the survival of both β -cells and ECs during short term culture. Therefore, the aims of this study were to determine whether preculturing islets with MSCs improves islet function and maintains EC number *in vitro*. We also investigated whether preculturing islets with MSCs improves islet transplantation outcome.

7.2 Methods

7.2.1 Measurement of basal and glucose stimulated insulin secretion *in vitro*

Preliminary experiments were designed to establish whether MSCs have beneficial effects on islet function and if so, whether direct cell-cell interactions between islets and MSCs are needed. To study this, we used a direct-contact or transwell co-culture system (as described above). Islet function was assessed using static incubations of islets and measuring insulin secretion. Islets were washed with fresh RPMI and pre-incubated for 2 hr in RPMI containing low glucose (2mmol/l). To measure basal and glucose stimulated insulin secretion, groups of three islets were then transferred to 1.5ml eppendorf tubes and incubated in 300µl of 2mmol/l or 20mmol/l glucose Gey and Gey buffer respectively, at 37°C for 1 hr. At the end of the incubation period, the islets were centrifuged at 380 x g for 2 min, at 4°C. 200µl of the supernatant was then removed, added to 800µl of borate buffer in 1.5ml eppendorfs and stored at -20°C until analysis. The insulin content of the incubation medium was then assessed by radioimmunoassay (see section 2.10.4). In these preliminary investigations, MSCs were shown to enhance islet function when using the direct-contact co-culture system, whereas there was no beneficial effect seen with the transwell system. Following these preliminary investigations, islets were precultured using the direct contact co-culture system only for all other *in vitro* experiments and transplantation studies and the transwell co-culture system was not investigated further, as we saw no beneficial effect of MSCs using the transwell system.

7.2.2 Measurement of insulin content

To determine the insulin content of islets, 10 islets in triplicate were picked for each condition, washed twice in PBS, before adding 200µl of acidified ethanol (absolute ethanol: deionised water: concentrated HCL in the ratio of 52: 17:1). Samples were then sonicated on ice, for 3x 5 second pulses, output 10-14 and insulin extracted overnight at 4°C. Samples were stored at -20°C until analysis. Each sample was diluted with borate buffer to an appropriate concentration for the detection limits of the radioimmunoassay. The insulin content of each sample was then measured by radioimmunoassay (see section 2.10.4).

7.2.3 Direct contact islet-MSC co-culture system

Approximately 200,000 kidney-derived MSCs of passage 7-12 were seeded into Nunclon™ 35mm 6 well plates, forming a confluent monolayer of cells within 12 hr. MSCs were cultured in 3ml DMEM supplemented with 1% vol/vol penicillin/streptomycin solution (Gibco BRL, Gaithersburg, MD, USA) 10% (vol./vol.) FCS (3ml/well), L-glutamine and incubated at 37°C in a humidified atmosphere containing 5% CO₂. The medium was changed after 24 hr, with removal of non-adherent cells. 100 freshly isolated islets were then added to each well and the medium was replaced with 3ml/well RPMI supplemented with 1% vol/vol penicillin/streptomycin solution (Gibco BRL, Gaithersburg, MD, USA) 10% (vol./vol.) FCS. Control islets (islet-alone) were cultured alone as groups of 100, in RPMI medium, in 35mm non-adherent tissue culture Petri dishes. The RPMI media was changed after two days for both culture groups and a total culture period of three days was used for all experiments. After three days direct co-culture with MSCs, the islets had formed loose attachments with the MSCs, but could be removed for *in vitro* analysis of islet function or transplantation experiments, by gently pipetting.

7.2.4 Transwell islet-MSC co-culture system

Approximately 200,000 kidney-derived MSCs of passage 7-12 were seeded into Nunclon™ 6 well plates and cultured as with the direct contact co-culture system for the first 24 hr before addition of islets. The medium was changed after 24 hr, with removal of non-adherent cells. Cell culture inserts with a semi-permeable membrane, pore size of 1.0µm (Becton Dickinson, NJ, USA) were inserted and 100 freshly isolated islets added into the inserts. The medium was replaced with RPMI supplemented with 1% vol/vol penicillin/streptomycin solution (Gibco BRL, Gaithersburg, MD, USA) 10% (vol./vol.) FCS (3 ml/well). Control islets (islet-alone) were cultured in the same way, but using 35mm non-adherent petri dishes. The RPMI media was changed after two days and a total culture period of three days was used.

7.2.5 Transplantation

Mice were transplanted with either 150 islets cultured alone or precultured on a confluent monolayer of kidney-derived MSCs (direct contact co-culture), for three

days. Islets were separated from the MSCs prior to transplantation. The number of transplanted islets was chosen to act as a minimal islet mass, intending to reverse hyperglycaemia in only a proportion of diabetic recipients. The surgical procedure for transplantation was the same as that detailed in section 2.3.3, with mice in both groups being transplanted with islets that had been centrifuged/pelleted into the PE50 polyethylene tubing.

7.2.6 Analysis of isolated islets and islet grafts

All immunostaining was performed using the 3,3'-diaminobenzidine (DAB) developing system. Images were taken using an Olympus BX40 light microscope and Q imaging micropublisher 5.0 RTV camera, using Q capture Pro (v.5.1) software. Adobe Photoshop Elements 4.0 and Image J computer software (<http://rsbweb.nih.gov/ij/>) were used for determining β -cell number, islet area and vascular density (number of ECs per square millimetre).

7.2.6.1 Freshly isolated and cultured islets

The vascular density was defined as the number of intraislet ECs (CD34+ or CD31+ cells) per islet area. β -cell number was determined by counting the number of cells staining positively with insulin antibodies. For each time point (freshly isolated or three day cultured islets) 15-50 islets were analyzed from each mouse.

7.2.6.2 Transplanted islets

For each animal ≥ 5 tissue sections were evaluated. The tissue was sectioned so that the analysis was done throughout the graft to ensure that the mean value was obtained for the vascular density of transplanted islets and that any regional differences were taken into account. The number of ECs was counted at a magnification of x400. ECs in the endocrine and non-endocrine components of the graft were counted separately. The area of the graft was determined using Image J software and the vascular density (number of ECs/mm²) was determined. The demarcation of the islet graft was taken as the area of endocrine tissue in between the renal parenchyma and the kidney capsule.

7.3 Results

7.3.1 Measurements of insulin secretion *in vitro*

Preliminary studies were designed to investigate the effects of preculturing islets with MSCs upon islet function *in vitro* using a direct-contact or transwell co-culture system, in order to determine the best co-culture system for subsequent transplantation studies and *in vitro* studies. Islet function *in vitro* was assessed using basal and glucose stimulated insulin secretion assays. Using a direct co-culture system, it was shown that MSCs had no effect on insulin secretion in the presence of sub-stimulatory glucose concentration (2mmol/l), but potentiated insulin secretion at 20mM glucose (Figure 7.1a). To determine the importance of direct cell-cell contact for the MSC-dependent improvements in insulin secretory capacity, the same experiment was repeated using a transwell co-culture system. This system utilised a semi-permeable membrane to separate the islets from MSCs. After a three day co-culture period, MSCs had no effect of insulin secretion at 2mmol/l glucose and in contrast to their effects using a direct co-culture system; MSCs had no effect on glucose stimulated (20mmol/l) insulin secretion (Figure 7.1b). Thus, for all subsequent experiments both *in vitro* and *in vivo*, islets were precultured with MSCs using the direct contact co-culture system and compared to control islets cultured in the absence of MSCs. The transwell co-culture system was not investigated further.

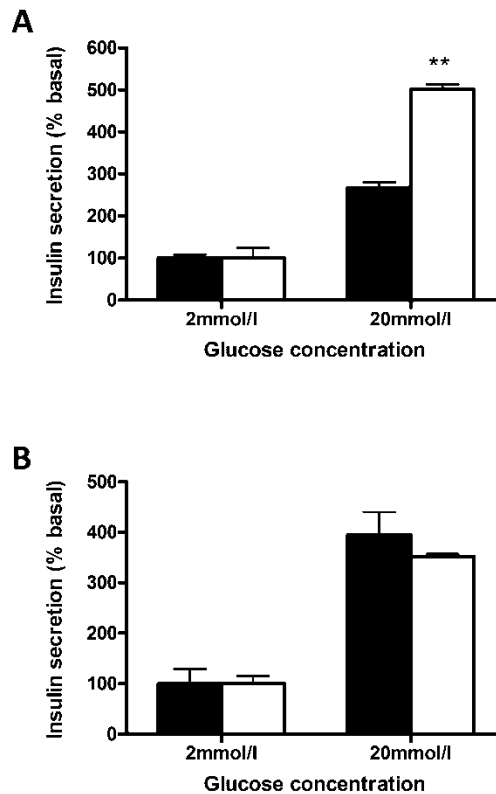


Figure 7.1 Insulin secretion *in vitro*. **A.** Direct-co-culture system: Insulin release at 2mmol/l glucose and 20mmol/l glucose, of 9 replicates of triplicate ICR mouse islets cultured (direct co-culture) for 3 days with 200,000 MSCs (white bars) or without MSCs (black bars), ** $p < 0.002$ vs absence of MSCs at the same glucose concentration, Two-way ANOVA with Bonferroni post hoc test, $n=5$. **B.** Transwell co-culture system: Insulin release at 2mmol/l glucose and 20mmol/l glucose, of 9 replicates of triplicate ICR mouse islets cultured using transwell co-culture system for 3 days with 200,000 MSCs (white bars) or without MSCs (black bars), $p > 0.05$ when comparing culture groups at the same glucose concentration, Two-way ANOVA with Bonferroni post hoc test, $n=5$.

7.3.2 Islet-MSC direct co-culture system

Freshly isolated islets (Figure 7.2a) were cultured either alone for three days (Figure 7.2b) or added to kidney-derived MSCs of passage 7-12, which had been seeded 24 hr beforehand (Figure 7.2c) and then co-cultured for three days using a direct-contact system (Figure 7.2d). The MSCs were 90-100% confluent at the time of adding freshly isolated islets. Within 24 hr, islets formed loose attachments with the MSCs that had formed a monolayer on the bottom of the culture dish and these islet-MSC interactions remained throughout the three day culture period.

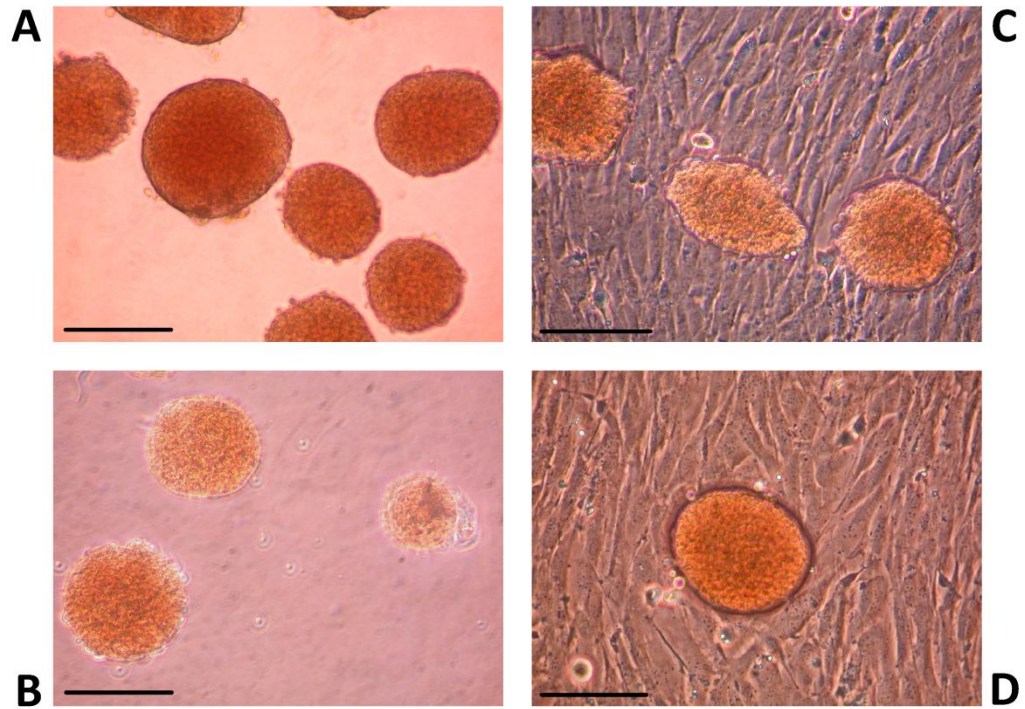


Figure 7.2 Islet-MSC co-culture system. Phase-contrast photomicrographs of islet-MSC co-culture system. **A.** Freshly isolated islets **B.** Three day cultured islets **C.** Freshly isolated islets added to pre-seeded MSCs **D.** Three day MSC co-cultured islets, scale bars are 100µm.

7.3.3 Graft function

Preculture of islets with MSCs using the direct-contact co-culture system improved graft function as shown in Figure 7.3. Average blood glucose concentrations were significantly lower at 7, 14, 21 and 28 days post transplantation in recipients of MSC precultured islets, compared to recipients of islets cultured alone (Figure 7.3a). At one month, all mice in the MSC precultured islets transplant group had cured compared to only 40 percent of mice transplanted with islets cultured alone (Figure 7.3b). The average time to cure was 16 ± 3 days for mice transplanted with MSC precultured islets, with islet-alone mice taking considerably longer (28 ± 1 , $p < 0.001$, $n=10$). The median time to cure was 14 and 28 days for islet + MSC and islet-alone cultured islet recipients respectively. There were no differences in the starting body weight of mice in either transplant group (27.1 ± 0.6 and 26.7 ± 0.7 g for recipients of islets cultured alone and MSC precultured islets respectively, $p = 0.98$, $n=10$, Student's t test). However, mice transplanted with MSC precultured islets had a significantly higher weight at 28 days than mice transplanted with islets cultured alone (27.4 ± 0.6 and

$25.4 \pm 0.6\text{g}$, $p = 0.04$, $n=10$, Student's t test), reflecting the better glycaemic control of mice transplanted with MSC precultured islets. At one month all cured mice were given an IPGTT. Glucose tolerance was similar for mice in both transplant groups (Figure 7.3c). All cured mice in both transplant groups were nephrectomised, with all mice reverting to hyperglycaemia within two days (Figure 7.3d).

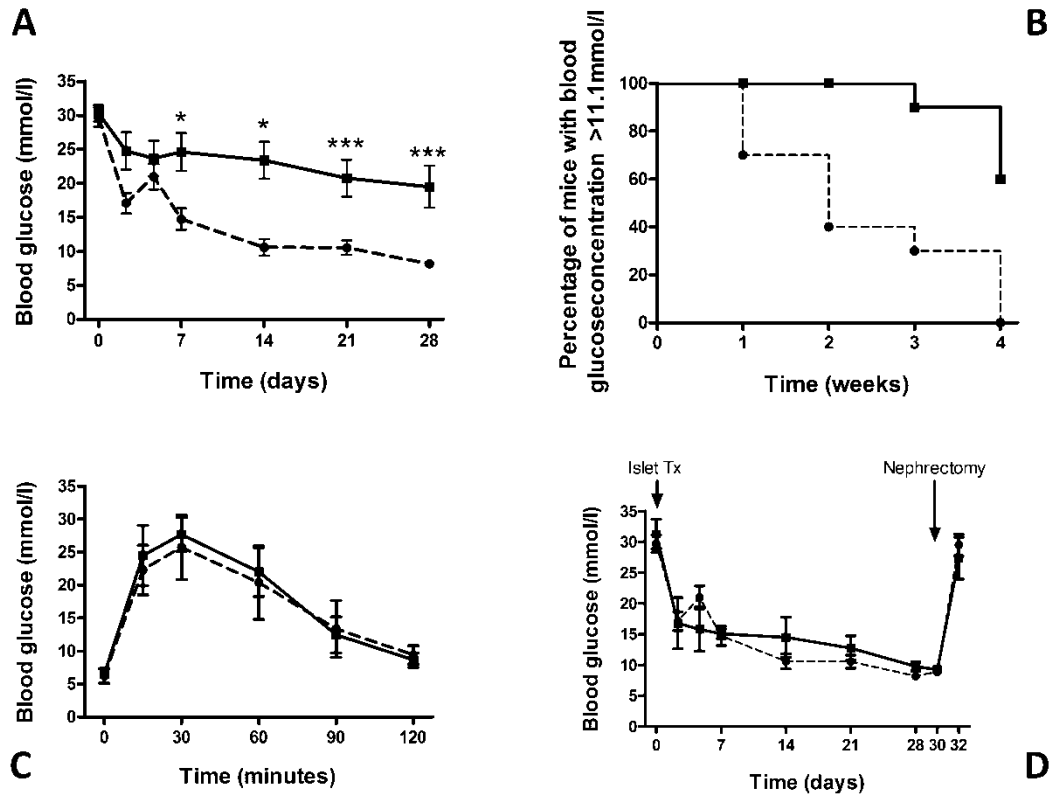


Figure 7.3 Graft function **A.** Blood glucose concentrations of mice transplanted with islets cultured alone for three days (continuous line) or islets precultured with MSCs (using the direct contact co-culture system) for three days (dashed line); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs mice transplanted with MSC precultured islets (RM ANOVA with Bonferroni post hoc test, $n=10$ for both transplant groups). **B.** Percentage of mice remaining diabetic (blood glucose concentration >11.1 mmol/l) after transplantation as in A, $p = 0.001$, Kaplan–Meier, $n=10$ for both transplant groups. **C.** IPGTTs in all cured mice at one month post transplantation, $p > 0.05$, two-way RM ANOVA with Bonferroni post hoc test, $n= 4-10$. **D.** Blood glucose concentrations of cured mice only after islet transplantation (day 0) and after removal of the graft bearing kidney at one month post transplantation ($n=4-10$).

7.3.4 Endothelial cell density and β -cell number in cultured islets

To try to establish the mechanism by which preculturing islets with MSCs improved transplantation outcome, we determined the extent to which MSCs were able to

maintain intranslet EC survival during the culture period prior to transplantation. CD34 and CD31 antibodies were used to immunostain consecutive sections of islets cultured alone, or with MSCs for three days using a direct contact co-culture system. The number of CD34+ ECs in islets cultured alone was extremely low (Figure 7.4a), confirming previous results (as shown in Figure 3.6). Similarly, there was a distinct lack of CD34-positive ECs in MSC precultured islets (Figure 7.4b). We also immunostained islet sections from each culture group with CD31 antibodies as an additional EC marker, to determine whether both CD34 and CD31 EC markers showed a similar reduction with time in culture. The number of CD31+ ECs present in both islets cultured alone (Figure 7.4c) and MSC precultured islets (Figure 7.4d) was also very limited. The density of CD34+ and CD31+ ECs was quantified by counting the number of CD34+ and CD31+ cells respectively per islet area. There were no differences in the density of either CD34+ or CD31+ ECs between culture groups, however there was differential expression of CD34+ and CD31+ ECs in islets cultured alone and a trend towards enhanced numbers of CD31+ ECs in MSC co-cultured islets (Figure 7.4e). The number of β -cells in islet sections from both culture groups was also quantified. Immunohistochemical analysis indicated that preculturing islets with MSCs partially prevented the loss of β -cells that was previously observed during culture (see Figure 3.3), with a significantly higher number of insulin-positive β -cells observed in MSC precultured islets compared to islets cultured alone for three days (83.7 ± 5.1 and 54.4 ± 6.5 β -cells per islet section, $n=4$, $p=0.03$, Student's t test).

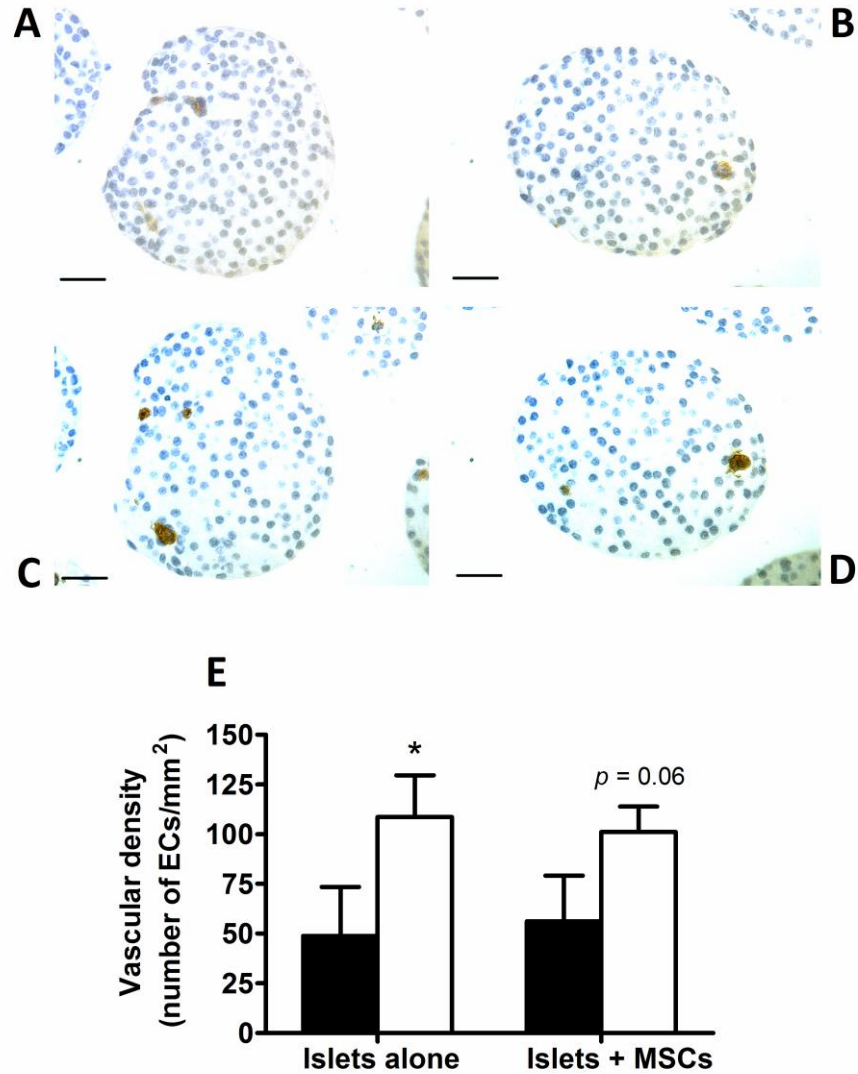


Figure 7.4 Vascular density of cultured islets. Consecutive sections of islets cultured alone (a, c) and islets precultured with MSCs for three days using a direct co-culture system (b, d), immunostained with CD34 antibodies (a, b) and CD31 antibodies (c, d), as markers of microvascular ECs. E. CD34+ EC density (black bars) and CD31+ EC density (white bars) of islets cultured alone or with MSCs, Two-way ANOVA with Bonferroni post hoc test, * $p < 0.05$, $p = 0.06$ vs same sections stained with CD34 antibodies in same culture group, $n = 4$.

7.3.5 Vascular density of islet grafts

Although preculturing islets with MSCs had no positive effect on intraislet ECs *in vitro*, we wanted to establish whether there was any influence of MSC preculture on the extent to which islet grafts revascularise *in vivo*; to determine whether this could be a potential mechanism for the improved graft function observed in the recipients of MSC precultured islets. CD34 antibodies were used to immunostain microvascular endothelium in grafts consisting of islets cultured alone (Figure 7.5a) and islets

precultured with MSCs using the direct contact co-culture system, for three days (Figure 7.5b). There were no major differences between transplant groups, in terms of the distribution of ECs within the endocrine tissue. Quantification of ECs within endocrine and non-endocrine components of the grafts showed that the vascular density of the endocrine tissue was similar between transplant groups, as shown in Figure 7.5c. There was a markedly higher EC density in the non-endocrine tissue surrounding the islet tissue, for grafts consisting of islets cultured alone (Figure 7.5c), compared to grafts consisting of MSC precultured islets. The percentage of non-endocrine tissue was lower in grafts consisting of islets cultured alone than those consisting of MSC precultured islets (17.6 ± 3.5 and 30.1 ± 2.7 percent for grafts consisting of islets cultured alone and MSC precultured islets respectively, $n=4$, $p = 0.03$, Student's t test).

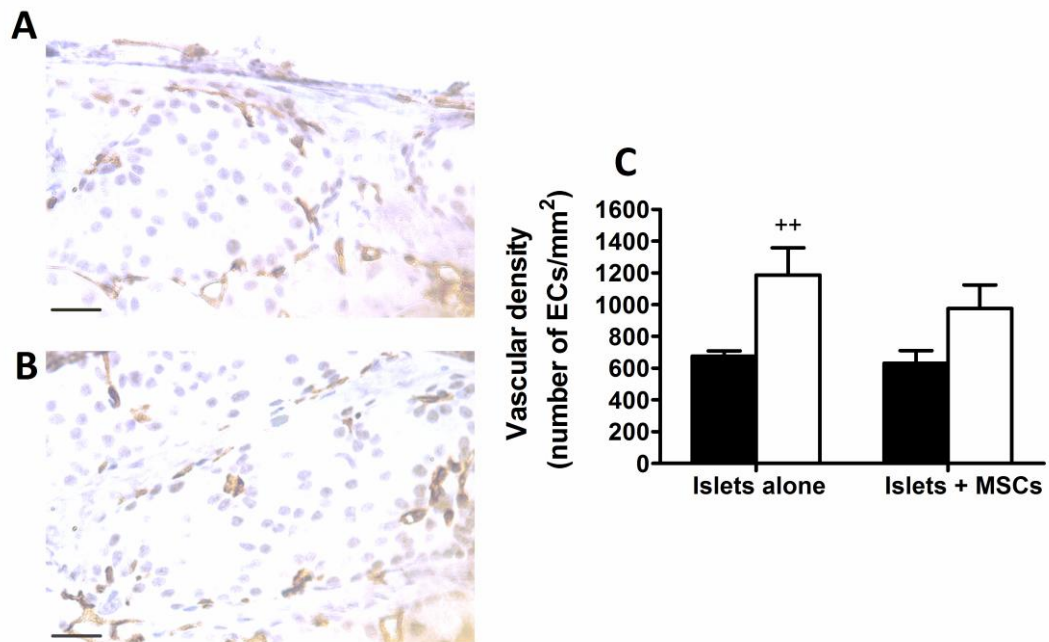


Figure 7.5 Vascular density of islet grafts. CD34 staining of microvascular ECs in one month grafts consisting of islets cultured alone (a) or islets precultured with MSCs using the direct-contact system, for three days (b). Images are representative of four animals in each transplant group, original magnification x400, scale bars are 25µm. C. Vascular density of endocrine components (black bars) and non-endocrine components (white bars) in one month grafts consisting of islets cultured alone or MSC precultured islets, ++ $p < 0.01$ vs endocrine tissue within same transplant group, Two-way ANOVA with Bonferroni post hoc test, $n=4$.

7.3.6 Islet number, area and insulin content

Having shown that preculturing islets with MSCs has no positive effect on intraislet EC survival *in vitro* or islet graft revascularisation *in vivo*, we wanted to investigate the extent to which MSCs have the capacity to maintain functional β -cell mass during the culture period prior to transplantation, as a potential mechanism for the improved graft function seen in recipients of MSC precultured islets. Islet number, area and insulin content were measured for control islets cultured alone and islets precultured with MSCs using the direct contact co-culture system, for three days. The number of islets was counted in each culture group three days post isolation, to determine the extent to which whole islets were lost *in vitro*, in addition to the functional β -cell mass of individual islets. At the time of isolation 100 islets were counted into each petri dish. After three days, the number of islets was reduced by less than ten percent, with no differences in islet number observed between culture groups (Figure 7.6a). Islet area was also similar between culture groups after three days (Figure 7.6b). The insulin content of isolated islets decreased by 65% after three days culture in the absence of MSCs (Figure 7.6c). In separate experiments, it was shown that preculturing islets with MSCs prevented this decline in insulin content, with a significantly higher (47% higher) insulin content observed in MSC precultured islets than islets cultured alone for three days (Figure 7.6d).

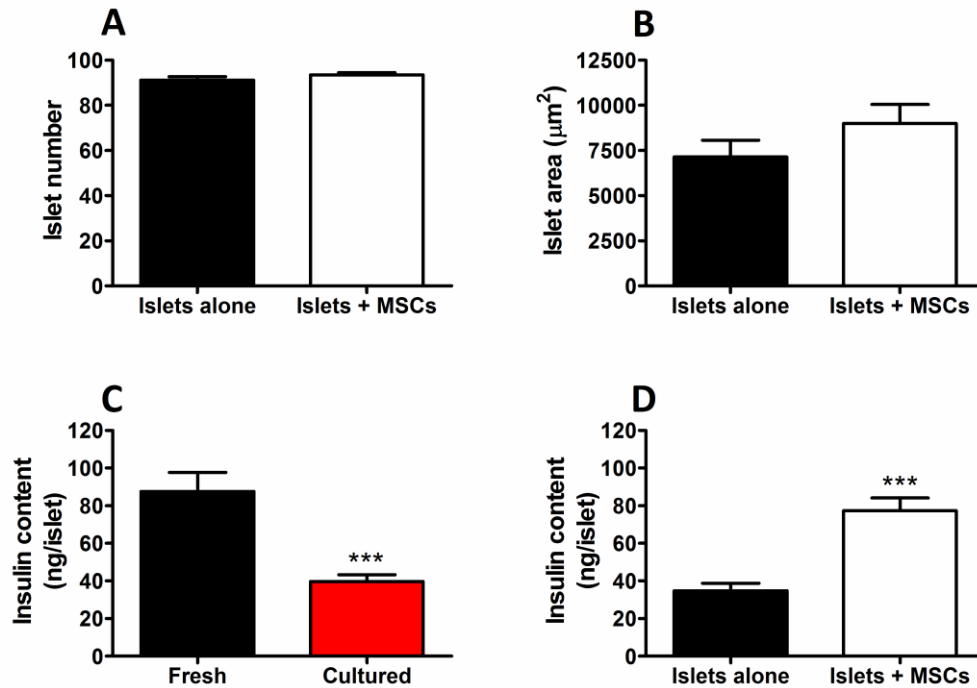


Figure 7.6 Islet number, area and insulin content. **A.** Control islets cultured alone (black bars) and islets precultured with MSCs using the direct contact co-culture system (white bars) were counted three days after isolation, with an initial number of 100 islets per culture dish, mean of islet number \pm SEM, $n=16$, $p = 0.23$, Student's t test. **B.** Islet area of control islets cultured alone (black bars) and islets cultured with MSCs using the direct contact co-culture system (white bars) after three days, $n=4$, $p = 0.24$, Student's t test (same islets as those analysed for EC number, as in Figure 7.4). **C.** Insulin content of fresh islets (black bars) and three day cultured islets in the absence of MSCs (white bars), $n = 15$ groups of 10 islets from 5 separate isolations, $p = < 0.0001$ vs freshly isolated islets, Student's t test. **D.** Insulin content of control islets cultured alone (black bars) and islets precultured with MSCs using the direct contact system for three days (white bars), $n=15$ groups of 10 islets from 5 separate isolations, $p = < 0.0001$ vs islets cultured alone, Student's t test.

7.4 Discussion

Using a syngeneic minimal islet mass model, it was demonstrated that preculturing islets with MSCs was able to improve both the number and rate at which mice achieved normoglycaemia by one month. Notably, the curative capacity of MSC precultured islet grafts was comparable to that of fresh islets, which were previously shown to produce superior transplantation outcomes to that of islets cultured alone (see Figure 3.1), in accordance with other studies (King et al., 2005, Olsson and Carlsson, 2005). Importantly, the number of islets transplanted in the previous experiment was 200, as opposed to the more stringent minimal islet mass of 150 islets used in the current study. This suggests that preculturing islets with MSCs essentially reduces the number of islets required to achieve normoglycaemia.

A number of recent studies have suggested that co-culture with MSCs may improve islet viability and function and thus improve the quality of islet material used for transplantation. A recent report demonstrated that culturing islets with MSC-conditioned media prior to transplantation improved graft function *in vivo*, which was attributed to MSC-derived trophic factors improving islet quality, survival and function (Park et al., 2010). In addition to the positive paracrine influences MSCs are reported to have on adjacent cells through the secretion of soluble mediators (Xu et al., 2008, Park et al., 2009, Figliuzzi et al., 2009, Boumaza et al., 2009, Sordi et al., 2010, Ito et al., 2010, Karaoz et al., 2010), there is also evidence to suggest that direct cell-cell contact is important for their beneficial effects (Jung et al., 2011). In our preliminary *in vitro* studies, we observed superior islet function when islets were co-cultured with MSCs using a direct contact co-culture system compared to islets cultured alone. However, in contrast to the results of Park et al (Park et al., 2010), MSCs did not exert a positive influence on glucose stimulated insulin secretion, when islets were cultured together with MSCs using a transwell co-culture system. This suggests that direct islet-MSC contact is important for the enhanced insulin secretory response observed in our *in vitro* studies, which is consistent with a recent study by Jung et al, who showed that physical contact was more effective in maintaining islet survival, morphology and insulin secretory capacity than the presence of MSC-secreted trophic factors alone (Jung et al., 2011). Therefore, a direct contact co-culture system was utilised, in which the islets were cultured on a confluent monolayer of MSCs for the transplantation studies and all subsequent *in vitro* studies. This system ensures that there is direct cell-cell contact between the islets and MSCs, but also that the islets are exposed to high concentrations of any soluble trophic molecules secreted by the MSCs.

There is some evidence to suggest that cell density or the ratio of MSCs to islet cells may be important in determining the efficacy of MSCs in improving islet function and survival (Longoni et al., 2010), so the ratio of islets: MSCs was kept constant at 100 islets: 200,000 MSCs throughout this study. This corresponds to a 1:1 cell ratio (where an islet is considered to consist of approximately 2000 cells). The direct contact co-culture system used did not adversely affect the morphology and

architecture of cultured islets. Furthermore, there were no obvious signs of 'contaminating' MSCs surrounding the islets, indicating that it possible to retrieve islets from this culture system, whilst essentially excluding MSCs from the islet preparations used for both the transplantation studies and *in vitro* analysis of islet function.

All cured mice in both transplant groups were nephrectomised at one month to rule out any possibility that mice had cured due to β -cell regeneration in the endogenous pancreas. Within two days after removal of the graft-bearing kidney, all mice did indeed revert to hyperglycaemia, demonstrating that the positive effects of MSCs were related to improved function of the islet graft. In accordance with our previous observations (see Figure 4.3), glucose tolerance was similar between cured mice in both transplant groups, suggesting that preculturing islets with MSCs increases the curative capacity of cultured islets, but does not increase functionality after reversal of hyperglycaemia, compared to grafts consisting of islets cultured alone.

In Chapter Four, it was demonstrated that co-transplantation of islets with MSCs has a beneficial effect on the treatment of diabetic hyperglycaemia, with associated improvements in the anatomical remodelling and vascularisation of the graft. Thus, we hypothesised that preculturing islets directly with MSCs might improve the quality of cultured islets for transplantation with regard to their angiogenic potential and function. It is well established that the islet isolation procedure disrupts the islet vasculature and therefore the islets are dependent on their oxygen supply by diffusion during the subsequent culture period, which particularly threatens the survival of centrally located β -cells (MacGregor et al., 2006). In previous experiments, we characterised the extent to which β -cells and intra-islet ECs were lost during culture to determine whether the quality of islets prior to transplantation was affected by a three day culture period and whether this may contribute to the better transplantation outcome with fresh islets observed in the previous study (see Chapter Three). Consistent with other reports, we also showed that maintenance of islets in culture was associated with a decline in the numbers of both β -cells (Kin et al., 2008, Wang and Rosenberg, 1999) and ECs (Parr et al., 1980, Nyqvist et al., 2005, Olsson et al., 2006) within the islets,

either or both of which may be responsible for the reduced efficacy of cultured islets as transplantation material.

The survival of intranslet ECs may be important as studies have shown that donor ECs contribute to the formation of functional blood vessels within the transplanted islets (Linn et al., 2003b, Brissova et al., 2004, Nyqvist et al., 2005) and may also accelerate the rate of islet revascularisation *in vivo* (Nyqvist et al., 2011), which is important as enhanced vascularisation is associated with better graft function (Johansson et al., 2009c). A number of studies have also suggested that ECs can exert beneficial paracrine effects on adjacent β -cells (Linn et al., 2003b, Lai et al., 2005, Johansson et al., 2009c, Olerud et al., 2011). It is therefore likely that maintaining intranslet ECs would enhance β -cell survival. MSCs derived from a number of sources have been shown to secrete an array of trophic molecules (Park et al., 2009, Boumaza et al., 2009, Karaoz et al., 2010), including angiogenic factors (Figliuzzi et al., 2009, Sordi et al., 2010, Ito et al., 2010, Berman et al., 2010), which could potentially improve EC survival. A recent study also showed that direct contact co-culture with MSCs can increase the secretion of VEGF by the islets themselves, compared with control islets (Jung et al., 2011), which could potentially increase β -cell function and mass during the post transplantation period (Lai et al., 2005). Furthermore, MSC secreted anti-inflammatory molecules may help to enhance islet cell recovery from the detrimental effects associated with the isolation process (Wang and Rosenberg, 1999, Rosenberg et al., 1999, Paraskevas et al., 2000, Pileggi et al., 2001). Despite this, there was no evidence of any beneficial effect of direct islet-MSC co-culture on intranslet EC survival as shown by the dramatic loss of both CD34+ and CD31+ ECs following a three day culture period. Notably, the number of CD31+ ECs in both culture groups was higher than that observed for CD34+ ECs, indicating that there is a minor subset of CD31+CD34- ECs present after three days culture. Islet ECs are generally quiescent in adult rodents with a very low proliferative capacity *in vivo* (Johansson et al., 2006a), however, there is evidence to suggest that a small number of remnant ECs remain in cultured islets, with an angiogenic phenotype (Linn et al., 2006). CD31 has been shown to be expressed on proliferating ECs (Bencini et al., 1993), as well as cultured islet ECs (Nyqvist et al., 2005), whilst some studies have indicated that CD34 antibodies do not recognise the CD34 antigen in proliferating ECs, because it

may be down regulated and/or processed into a form that is not recognised by the antibodies (Fina et al., 1990). Therefore, it is possible the small numbers of CD31+CD34- ECs observed in three day cultured islets in this study, represent a proliferative population of ECs.

A number of reasons may account for the fact that MSCs were unable to preserve intraislet ECs during short term culture. The secretion of trophic factors was not determined in this study, so it is possible that our MSC population do not secrete the angiogenic factors that have been shown by others (Figliuzzi et al., 2009, Park et al., 2009, Boumaza et al., 2009, Sordi et al., 2010, Ito et al., 2010). Alternatively, the concentration of factors such as VEGF, HGF and IL-6 may be high, as with other reports, but not sufficient to prevent EC de-differentiation and death in the absence of any three-dimensional matrix support, as exists *in vivo*. In support of this hypothesis, two independent studies have shown that supplementation of the islet culture medium with typical angiogenic factors, including VEGF and FGF, had no significant effect on the dramatic loss of ECs in culture (Nykqvist et al., 2005, Olsson et al., 2006). It is possible that the intraislet ECs migrate out of the islet towards the higher concentration of angiogenic factors in the culture medium, as indicated by Olsson and colleagues (Olsson et al., 2006). It is also important to note that the effects of individual angiogenic factors are dependent on the microenvironment, but also the presence and absence of other angiogenic or indeed angiostatic factors (Linn et al., 2003b).

Another possible mechanism by which preculturing islets with MSCs may improve islet function *in vivo*, is by improving the angiogenic capacity of the implanted islets and therefore potentially enhancing the extent to which the transplanted islets are revascularised. However, no differences in EC distribution or density of the graft endocrine tissue were observed at one month, in contrast to the superior vascular engraftment previously shown in the MSC co-transplantation studies (see Figure 4.9 and Figure 4.10). Similarly to the previous MSC co-transplantation study, we observed large number of ECs in the non-endocrine parenchyma surrounding the islets within the grafts of islets cultured alone. The EC density within the non-endocrine stromal tissue was lower in the grafts consisting of MSC precultured islets,

which was associated with a significantly higher area of non-endocrine tissue compared to the grafts consisting of islets cultured alone. Thus in the current study the improved islet function observed both *in vitro* and *in vivo* after culturing islets with MSCs, appear to be independent of any positive effect on EC survival and/or islet graft revascularisation.

The most likely explanation for the improved outcomes of transplantations using MSC-precultured islets is that the MSCs improve islet survival and/or function during the pre-transplantation culture period. Our *in vitro* measurements clearly demonstrated that direct contact co-culture with MSCs potentiated glucose stimulated insulin secretion. These observations are in accordance with recent studies (Park et al., 2010, Lu et al., 2010, Jung et al., 2011), in which the superior secretory capacity of MSC precultured islets has been attributed to the MSC-dependent enhanced expression of β -cell specific genes, such as PDX-1 (Boumaza et al., 2009, Park et al., 2010) or to increased β -cell survival, via the up regulation of anti-apoptotic molecules including Bcl-2, XIAP and Bcl-xL (Park et al., 2010). We have demonstrated that islet insulin content was significantly higher for islets cultured with MSCs than that for islets cultured alone, indicating an enhanced functional β -cell mass and consistent with the MSC-dependent maintenance in β -cell numbers during culture. Notably, the insulin content of MSC precultured islets was comparable to that of fresh islets, indicating that MSCs are able to prevent the decline in insulin content that was shown to occur during a three day culture period, in agreement with previous studies (Mattsson et al., 2004, Olsson and Carlsson, 2005). A number of studies have emphasised the importance of the initially transplanted β -cell number for improved long term transplantation outcomes (Keymeulen et al., 1992, Tobin et al., 1993), in accordance with the improved *in vivo* performance of freshly isolated islets or MSC-precultured islets, in both of which the functional β -cell mass is maintained compared to cultured islets.

The findings of the current study are potentially of use in the clinical setting, where it may not always be possible to transplant freshly isolated islets for logistical reasons (Goss et al., 2002, Shapiro et al., 2003, Hering et al., 2004, Froud et al., 2005, Kin et al., 2008). Our experimental observations predict that preculturing human islets with

MSCs will maintain islet function during the pre-transplantation period. Furthermore, although reports of adverse events associated with stem cell co-transplantation strategies in humans are generally low (Berman et al., 2010), there are still risks associated (Amariglio et al., 2009, Thirabanjasak et al., 2010). Preculture of islets with MSCs *ex vivo* would reduce some of the safety concerns of treating patients with MSCs *in vivo*; either by systemic MSC infusions or islet-MSC co-transplantation. Co-culture of islets with MSCs prior to transplantation may therefore offer a simple and safe method for improving islet quality and transplantation outcomes.

8 Chapter Eight - General Discussion

There have undoubtedly been major advances within the clinical islet transplantation field with the development of the Edmonton protocol during the 1990s (Shapiro et al., 2000) causing increased enthusiasm for the potential of a more widespread application of allogeneic islet transplantation as a therapy for patients with type 1 diabetes mellitus (T1DM). Although patients transplanted using the Edmonton protocol undoubtedly had an improved quality of life resulting from improvements in hypoglycaemia, the longer term outcomes in terms of maintaining normoglycaemia, of allogeneic islet transplantation using the Edmonton protocol were disappointing (Ryan et al., 2005a), due to obstacles revolving around immunological rejection, inadequate engraftment, suboptimal function of the transplanted islets and extensive cell death during the post transplantation period. Substantial efforts have addressed many of these issues and improvements have continued since the development of the Edmonton Protocol, thereby ‘closing the gap’ between clinical allogeneic islet transplantation and whole organ pancreas transplantation, in terms of the efficacy of these treatments to render the patient insulin independent (Shapiro, 2011b). Although some centres have had success with single-donor islet transplantations (Hering et al., 2004, Posselt et al., 2010, Matsumoto, 2011), the large majority of patients require islets from multiple donors. Taken together with the shortage of human islet donors, this means that islet transplantation is still limited to a small subset of patients with severe hypoglycaemia unawareness, glycaemic lability or kidney transplant (which can be implanted prior to the islet transplantation or simultaneously).

In the studies presented here, we have shown that Mesenchymal Stem Cells (MSCs) have a number of therapeutic properties which enable them to essentially act as ‘islet helper cells’, enhancing the functional β -cell mass of cultured islets during the pre-transplant period and also improving morphological and vascular engraftment during the post transplantation period, in a co-transplant setting. As a consequence, preculturing islets with MSCs, as well as co-transplanting islets with MSCs, was associated with better curative capacity of islet grafts and enhanced glycaemic control in recipient mice, compared to islets cultured alone or transplanted alone,

respectively. Thus, these studies suggest that there are a number of clinical applications for using MSCs to improve transplantation outcome, which may help in the achievement of routine single-donor islet transplantation, thereby making better use of the human islets that are available. Additionally, these studies have shown that maintaining normal islet organisation and morphology at the implantation site is important for enhanced islet function. A number of alternative implantation sites to that of the intraportal site are currently under investigation. Thus, ongoing strategies to improve the implantation site of islets should take into consideration the detrimental impact that implanting islets as clusters potentially has on transplantation outcome.

8.1 Improving functional β -cell mass by optimising islet culture conditions

Post isolation islet survival is crucial for optimizing islet transplantation outcome and making it as efficient as possible. Whilst the studies presented here suggest that there are important benefits of using fresh as opposed to cultured islets, in support of other findings (King et al., 2005, Olsson and Carlsson, 2005), culturing islets is beneficial as it allows time for functional tests, as well as safety tests including microbiological and pyrogenic (endotoxin) tests. Furthermore, culturing islets is more practical as it allows time for arranging the logistics of patient admission to hospital.

Our initial studies are in support of previous observations regarding the loss of β -cell mass during short-term islet culture (Wang and Rosenberg, 1999, Keymeulen et al., 2006, Ichii et al., 2007, Kin et al., 2008). Additionally, our data are in accordance with other studies showing the dramatic and significant loss of intraislet ECs during culture (Parr et al., 1980, Nyqvist et al., 2005, Olsson et al., 2006). In our subsequent experiments, we showed that preculturing islets with MSCs was able to preserve functional β -cell mass, but had no beneficial angiogenic effect either *in vitro* or *in vivo*, in contrast to the enhanced vascular engraftment *in vivo* demonstrated using an islet-MSC co-transplantation model. These findings suggest that alternative strategies to maintain intraislet ECs *in vitro* are needed. Possible methods for doing so may involve the use of three-dimensional matrices, which provide the islet cells with extracellular matrix (ECM) components, thus better mimicking the native *in vivo* microenvironment. Future studies may also incorporate the use of MSCs in

combination with ECM components to maximise the trophic support for islets during the pre-transplant period.

Notably, our *in vitro* studies showed that direct contact of MSCs with islets was important for the MSC-dependent enhancement of islet function *in vitro*, in accordance with other studies (Jung et al., 2011). These findings emphasise the importance of ensuring direct islet MSC interactions in both the pre- and post-transplant period, which is an important consideration for future experiments, which should aim to optimise the efficacy of MSC-based strategies for improving islet transplantation outcome in a clinically relevant setting.

8.2 Maximising the therapeutic properties of MSCs administered *in vivo*

Site-specific differences regarding islet engraftment exist (Lau et al., 2007) and it is therefore important to consider the translational implications of the findings presented in this thesis. Notably, regardless of implantation site or any potential modification to the implantation site, it seems likely that the most efficient use of MSCs in terms of islet function and engraftment will ensure that they are localized to the same site as the islets. Recent studies have investigated the use of alternative implantation sites to that of the intraportal route of administration, because of the environmental stresses this site confers. Intramuscular transplantation has shown successful outcomes (Rafael et al., 2008) and the omentum seems to offer the advantage of being well vascularized and portally drained (Rajotte et al., 1994). At present, the intraportal route is routinely used for human islet transplantation, but experimental studies should also consider the possibility that alternative sites may be used in the future. Certainly for intraportal transplantation, strategies to optimize the formation and optimal composition of composite MSC-islets, would be needed to ensure the co-localisation of islets and MSCs. Alternative sites may offer some flexibility in terms of the way in which the MSCs should be administered, due to the fact that the islets are not dispersed throughout the vasculature at extrahepatic sites, potentially allowing for co-transplantation strategies similar to the one used underneath the kidney capsule in our studies. Although, it may well be that the use of composite MSC-islets is the best option at all sites because this method potentially maximises the number of MSC-islet interactions.

MSC-dependent immunomodulation depends upon both cell-cell contact and soluble factors (Fibbe et al., 2007). Thus, it seems likely that transplanting composite MSC-islets would maximize the beneficial effects of MSCs in terms of their immunomodulatory properties, as well as their capacity to improve islet function and engraftment. Furthermore, the use of composite MSC-islets may also improve the survival of the MSCs, as the islets provide a surface for MSCs to attach to, which may prolong the time period over which MSCs exert their beneficial effects during the post transplantation period. At present, little is known about the lifespan of MSCs and this information will undoubtedly be valuable for determining the efficacy of additional MSC doses during the post transplantation period. Future studies should aim to track the fate of MSCs, by tagging them with a marker such as Green Fluorescent Protein (GFP). When MSCs are infused intravenously, there is evidence to suggest that some of the cells do indeed migrate to the islets (Sordi et al., 2005), however it is likely that some of the administered MSCs are likely to migrate to other sites, which complicates the issue of dosing and reduces the potential for direct islet-MSC interactions. Nevertheless, Berman and colleagues have demonstrated that repeated MSC infusions can delay islet graft rejection in non-human primates (Berman et al., 2010), although other studies have indicated that additional MSC doses have no therapeutic effect (Ezquer et al., 2008). Future studies should address ways in which MSCs should be administered at clinically relevant sites, including the intraportal route of administration, MSC dosing and whether or not repeated dosing is beneficial for transplantation outcome.

8.3 Should MSCs be used for preculturing islets, co-transplantation strategies, or both?

Our *in vivo* studies showed that preculturing islets with MSCs improved the curative capacity of minimal mass islet grafts, as did our islet-MSC co-transplantation method; raising the important question as to which strategy is the best for clinical allogeneic islet transplantation? The presence of MSCs at the graft site, particularly during the immediate post transplantation period is likely to be of importance, since MSCs have been shown to secrete a number of cytoprotective factors that could reduce hypoxia-associated cell death. Much of the progressive loss of β -cells after transplantation can

be attributed to chronic allograft rejection, recurrent autoimmunity, β -cell toxicity from the immunosuppressive drugs, as well as the immunosuppression related loss of β -cell replicative capacity. Thus, although MSC preculture and co-transplantation both improved islet graft function in our syngeneic transplantation model, it seems likely that in an allogeneic setting where MSCs have additionally been shown to exert potent immunosuppressive functions when administered *in vivo*, MSC co-transplantation may represent the best strategy for increasing the longevity of transplanted islets by reducing/preventing the loss of islets through recurrent autoimmunity and allograft rejection. However, future studies should verify the safety of MSC co-transplantation strategies, as well as MSC infusions, before we can fully determine what the best translational application of MSC based therapies should be.

The major safety concerns related to the use of MSCs are that of their potential to develop chromosomal aberrations resulting in oncogenic transformation (Tolar et al., 2007, Li et al., 2007), particularly after prolonged population doublings (Miura et al., 2006). In addition, ectopic formation of unwanted tissues, such as bone, as shown in a recent study in rodents (Duprez et al., 2011) is a concern. Whilst there have been reports of malignant transformation following islet-MSC co-transplantation in mice (Fiorina et al., 2009), tumour formation has not been demonstrated to be a major problem in current clinical trials using MSC based therapies for the treatment of autoimmune diseases. At present, there is little information regarding the safety of MSCs in humans in the context of T1DM. However, an ongoing phase II clinical trial conducted by Osiris therapeutics in partnership with the Juvenile Diabetes Research Foundation (JDRF), has shown that infusions of Prochymal, an adult MSC formulation, has shown no differences in adverse events compared to placebo at one year, when administered to patients with recent onset T1DM (http://www.osiris.com/clinical_prochymal_t1dm.php). Clearly, the long term implications of islet-MSC co-transplantation strategies are yet to be determined and more experimental, preclinical and clinical studies are needed. However, there have certainly been positive outcomes of clinical trials using MSCs for the treatment of other autoimmune disorders, such as graft versus host disease (GVHD) (Le Blanc et al., 2004, Le Blanc et al., 2008), allowing for an optimistic outlook upon their use for human islet transplantation.

A greater understanding of MSC biology is needed before the long-term risks of MSC-based therapies can be fully determined. Important factors requiring further investigation include the effect of passage number upon efficacy and safety (Abdi et al., 2008), differences relating to MSC dose and timing of dose, route of administration, source and whether or not repeated infusions should be administered acutely or over a prolonged time period. Although MSCs from a number of sources have been shown to promote islet graft function, engraftment, as well as exerting positive effects on the host's immune system, it is important to consider the practicalities of obtaining clinically relevant numbers of MSCs from any given source. Accessible sources of MSCs include adipose tissue from fat biopsies, exocrine pancreas tissue, umbilical cord, or bone marrow (BM), thus specific attention should be given to these MSC subsets for translational purposes in future studies.

The finding that preculturing islets with MSCs has the potential to maintain functional β -cell mass is of equal importance to the positive outcomes achieved with islet-MSC co-transplantation, as it suggests that this may be a strategy either alone or in combination with islet-MSC co-transplantation strategies for increasing the efficiency of islet transplantation.

In summary, it seems likely that preculturing islets with MSCs combined with MSC co-transplantation represents the most effective strategy for enhancing the efficiency of islet transplantation, helping to enable the more widespread application of islet transplantation as a therapy for T1DM.

8.4 Translational prospects for the future

For the majority of patients with T1DM, who do not suffer from life-threatening hypoglycaemia and who have reasonable metabolic control, the 'costs' of allogeneic islet transplantation do not currently outweigh the benefits. In particular, disadvantages of islet transplantation include the need for chronic life-long immunosuppression, associated with unwanted side effects and deleterious effects on long term graft function and survival, meaning that repeated islet infusions are required to maintain insulin independence. In this context, our data and those from

other groups suggest that MSC co-transplantation may increase the longevity of islet grafts through improved islet survival, engraftment and function, as well as the capacity for MSCs to prevent islet loss through immune responses to the graft. MSCs may potentially reduce/prevent the need for immunosuppression, thereby making allogeneic islet transplantation a more attractive treatment alternative for the majority of patients who do not have brittle diabetes, for whom at present the need for chronic immunosuppression means that insulin therapy is the preferred treatment.

Unfortunately however, the potential supply of islets from deceased human donors will never be sufficient to treat all patients worldwide with T1DM. Therefore, although the achievement of routine single-donor islet transplantation will undoubtedly be beneficial with regards to the number of people who can be given an islet transplant; it is clear that we are also in need of alternative β -cell sources to enable the more widespread application of islet transplantation as a therapy for T1DM.

A number of alternative β -cell sources are under investigation for β -cell replacement strategies. In particular, strategies have focused on producing insulin producing cells (IPCs) from embryonic (Soria et al., 2000, Lumelsky et al., 2001) or adult stem cell subsets including MSCs (Prabakar et al., 2011, Yuan et al., 2010). An important benefit of adult stem cell-derived IPCs is that they may be derived from the recipient/patient, thereby negating the need for chronic life-long immunosuppression and unwanted side effects. However, problems associated with the use of embryonic or adult-derived IPCs include the need for these cells to respond adequately to secretagogues such as glucose, thereby maintaining blood glucose concentrations within the physiological range, in a controlled fashion without causing hypoglycaemia, as has been shown to occur in rodents (Kroon et al., 2008). Our islet-MSC co-transplantation studies indicate that enhanced β -cell function *in vivo* is associated with increased numbers of ECs within the endocrine tissue of the graft. This is in support of other studies, emphasizing the importance of the highly specialized islet microenvironment, which incorporates not only ECs, but also ECM components. Thus, although advances in stem cell research may potentially provide an unlimited supply of insulin producing cells (Kroon et al., 2008); these single cells

may not necessarily have the same functional capacity as that of β -cells within an islet. Thus, an alternative and promising β -cell source is that from porcine islets allowing for cross-species islet transplantation (xenotransplantation).

Xenogeneic islets obtained from porcine pancreata are considered to be one of the most promising alternative sources of insulin producing cells (Bottino et al., 2007). There are encouraging results from pig islet transplantations in diabetic non-human primates (Hering et al., 2006, Cardona et al., 2006), particularly when islets from genetically engineered pigs are transplanted (van der Windt et al., 2009), suggesting that there is great potential for the use of clinical islet xenotransplantation in the future. However, numerous hurdles prevent the widespread application of xenotransplantation at present, with a need for reducing the antigenicity of porcine islets, potential infections with pig endogenous retroviruses and the instant blood mediated inflammatory response (IBMIR) (Rood et al., 2006). Advances in cell encapsulation strategies, such as the development of multilayer nanoencapsulation technologies (Krol et al., 2006, Zhi et al., 2012) may help to prevent the IBMIR by preventing islet-blood interactions and prolonging the survival of transplanted porcine islets. Furthermore, it has been shown that coating the surface of islets with MSCs may reduce the islet-blood contact/interactions, thereby reducing islet cell death associated with the IBMIR (Duprez et al., 2011). There is also evidence to suggest that porcine BM-derived MSCs have the capacity to reduce blood glucose concentrations in diabetic pigs through enhanced islet size and number of islets within the endogenous pancreas (Chang et al., 2008), indicating that MSCs may have the potential to improve the outcome of islet xenotransplantation, as well as allogeneic human islet transplantation. Important questions regarding the source of MSCs in this instance would need to be addressed, but there is certainly potential for combining the use of porcine islet - MSC co-transplantation and encapsulation strategies to increase the number of patients with T1DM who may be given an islet transplant. Additionally, there is the possibility that MSCs could potentially be used to improve the functional β -cell mass of porcine islets during the pre-transplant period as we showed for rodent islets.

Another potential use of MSCs is that they may be administered to recent onset diabetics or prediabetic individuals; to prevent disease progression before overt diabetes develops. Although our studies do not support a role for MSCs to enhance β -cell regeneration in the endogenous pancreas, there is substantial interest in the potential for MSCs to prevent disease progression or indeed reverse diabetes through enhanced β -cell regeneration, both in experimental studies (Lee et al., 2006, Dong et al., 2008, Ezquer et al., 2008), but also clinical trials. It is noteworthy that our studies were conducted over a one month monitoring period, which taken together with the intrinsically low replicative capacity of islet β -cells (Cnop et al., 2010), suggests that there may have been insufficient time for any significant β -cell regeneration to occur. Moreover, STZ-administration resulted in a pancreatic insulin content of less than 1% that present in the endogenous pancreas, which does not reflect the clinical situation in which there may be as much as 10-20% of the endogenous β -cells present at the time of diagnosis (Kloppel et al., 1985). Thus, given that there is a shortage of donor human islets, the potential for MSCs to cause endogenous β -cell regeneration within the islets should not be excluded and deserves further attention.

In summary, the studies presented in this thesis have shown that MSCs have great potential using a number of approaches to improve islet function, as well as engraftment and transplantation outcome in diabetic mice. Additionally, the importance of maintaining islet organisation and morphology should be considered when transplanting human islets in an allogeneic setting. These findings may help in the routine achievement of single-donor islet transplantation, thereby increasing the number of patients who can be given an islet transplant. In addition, these findings may have important implications for maximising the efficiency of alternative β -cell replacement sources, which may potentially be used for the more widespread application of islet transplantation.

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List of abstracts and publications from this thesis

- Chloe Rackham, Pedro Chagastelles, Nance Nardi, Astrid Hauge-Evans, Peter Jones and Aileen King, 2011, Co-transplantation of mesenchymal stem cells maintains islet organisation and morphology in mice. *Diabetologia*, 54:1127–1135.
- Chloe Rackham, Nicola Buckner, Paramjeet Dhadda, Peter Jones and Aileen King, 2011, Mesenchymal stem cells improve islet function and revascularization. *The Review of Diabetic Studies*, 8 (1): 146 (Abstract).
- Chloe Rackham, Pedro Chagastelles, Nance Nardi, Astrid Hauge-Evans, Peter Jones and Aileen King, 2011, Co-transplantation with mesenchymal stem cells maintains islet architecture and improves transplantation outcome. *Diabetic Medicine*, 28 Suppl1: 14 (Abstract).
- Chloe L Rackham, Anshi A Dattani, Peter M Jones and Aileen JF King, 2010, Culture of islets decreases islet cell mass and endothelial cell number and is detrimental for transplantation outcome in mice. *Diabetic Medicine*, 27 Suppl 1: 38 (Abstract).